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<b>(21) International Application Number:</b> PCT/US99/15842 <b>(22) International Filing Date:</b> 13 July 1999 (13.07.99) <b>(30) Priority Data:</b> 09/122,441                      24 July 1998 (24.07.98)                      US <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US    09/122,441 (CON) Filed on    24 July 1998 (24.07.98) <b>(71) Applicant (for all designated States except US):</b> MEGAN HEALTH, INC. [US/US]; 3655 Vista Avenue, St. Louis, MO 63110 (US). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> ROLAND, Kenneth, L. [US/US]; 6555 Mardel Avenue, St. Louis, MO 63109 (US). <b>(74) Agents:</b> HOLLAND, Donald, R. et al.; Howell & Haferkamp, L.C., 7733 Forsyth, Suite 1400, St. Louis, MO 63105 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> LIVE ATTENUATED <i>SALMONELLA</i> VACCINES TO CONTROL AVIAN PATHOGENS <b>(57) Abstract</b> <p>A vaccine for protecting birds against infection by avian pathogenic gram negative microbes is disclosed. The vaccine is a recombinant <i>Salmonella</i> strain expressing O-antigen of an avian pathogenic gram negative microbe such as an <i>E. coli</i> strain that is pathogenic in poultry. The recombinant <i>Salmonella</i> strain also does not express <i>Salmonella</i> O-antigen. Methods of using the vaccine to immunize birds are also disclosed.</p>		

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LIVE ATTENUATED *SALMONELLA* VACCINES TO CONTROL AVIAN PATHOGENSBackground of the Invention

This invention relates generally to vaccines for poultry and other birds and, more particularly, to vaccines for protecting poultry and other birds against infection by avian pathogenic gram-negative bacteria.

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Description of the Related Art

Avian pathogenic *E. coli* (APEC) strains cause a number of related diseases in poultry and other birds, including air sacculitis, cellulitis, colibacillosis, coligranuloma, colisepticemia, Hjarre's disease, omphalitis, peritonitis, salpingitis, synovitis (Gross, W.B. in *Diseases of Poultry*, Calnek et al., eds., Iowa State University Press, p. 138-144, 1991; Messier et al., *Avian Diseases* 37:839-844, 1993). These diseases and other diseases caused by gram-negative avian pathogens can lead to increased rates of feed conversion carcass condemnation, or death of the animal, resulting in millions of dollars lost to the poultry industry each year. Norton, R. A. *Broiler Industry*, Feb. 1998, pp. 28-32.

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Contamination of poultry products by *Salmonella* is a significant source of *Salmonella* infection in humans, which causes gastroenteritis, and thus is a major public health concern. Oral administration of live cells from *S. typhimurium* strains

having attenuating deletions in the *cya* and *crp* genes has been shown to provide excellent protection against wild-type *Salmonella* challenge in chickens (Hassan et al., *Res. Microbiol.* 141:839-850, 1990; Hassan et al., *Infect. Immun.* 62:5519-5527, 1994) and systems for stable expression of heterologous antigens in such strains have  
5 been developed (Hone et al., *Microb. Pathog.* 5:407-418, 1988; Strugnell, et al., *Gene* 88:57-63, 1990; Galan et al., *Gene* 94:29-35, 1990; Nakayama et al., *Bio/Technology* 6:693-697, 1995).

APEC strains are represented primarily by only a few serotypes, O1, O2, O35 and O78 (Cloud et al., *Avian Dis.* 29:1084-1093, 1985, Glantz et al., *Avian Dis.*  
10 6:322-328, 1962; Gross, *supra*), while *Salmonella* serotypes most prevalent in poultry are in the B, C and D groups. O-serotypes of gram-negative bacteria such as *E. coli* and *Salmonella* are determined at the molecular level by the so-called O-antigen structure, also termed O-specific chain or O-polysaccharide (O-PS), which generally is comprised of varying lengths of polymerized identical sugar units anchored in the  
15 bacterial outer membrane as the outermost component of lipopolysaccharide (LPS) molecules (Helander et al., in *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, R.A. Meyers, ed., VCH Publishers, Inc., 1995).

Specific antibody against LPS O-antigen has been shown to be protective in mammalian models of extraintestinal *E. coli* infections in humans (Cryz et al.,  
20 *Vaccine* 13:449-453, 1995; Pluschke et al., *Infect. Immun.* 49:365-370, 1985) and LPS O-antigen has been recognized as a protective antigen for other gram negative pathogens (Ding et al., *J. Med. Microb.* 31:95-102, 1990; Michetti et al., *Infect. Immun.* 60:1786-1792, 1992; Robbins et al., *Clin. Infect Dis.* 15:346-361, 1992). In addition, several research groups have reported using attenuated *Salmonella* and  
25 *Salmonella-E. coli* hybrids as vaccine delivery vehicles for O-antigens of several human pathogens, including *Shigella sonnei*, *Vibrio cholerae*, and *Pseudomonas aeruginosa*. (Black et al., *J. Infect. Dis.* 155:1260-1265, 1987; Formal et al., *Infect. Immun.* 34:746-750, 1981; Pier et al., *Infect. Immun.* 63:2818-2825, 1995); (Morona et al., U.S. Patent No. 5,110,588). However, until the work described herein,  
30 immunization of poultry with live, attenuated *Salmonella* expressing an APEC O-antigen had not been reported.

LPS O-antigen made by *E. coli* and *Salmonella* bacteria is comprised of lipid A, an R-core oligosaccharide, and the O-specific polysaccharide (O-PS), which are

covalently linked in that order. Sugiyama et al., *J. Bacteriol.* 173:55-58, 1991. In *S. typhimurium*, synthesis of the R-core moiety is directed by the *rfa* locus and certain housekeeping genes such as *galE*, *galU*, and *pgi*, while O-PS synthesis is directed by the *rfb* gene cluster, which encodes enzymes involved in biosynthesis of the monomer  
5 sugar unit, and the *rfc* gene, which encodes the O-antigen polymerase responsible for the polymerization of the sugar unit into a high molecular weight polysaccharide chain. Sugiyama et al., *supra*.

One group investigating the genes required for synthesis of LPS O-antigen in *E. coli* O9 introduced a plasmid containing the *rfb* locus from *E. coli* O9 into *S.*  
10 *typhimurium* wild-type and mutant strains with defects in the *rfb*, *rfc*, or *rfe* loci and reported that the wild-type strain containing the plasmid expressed LPS specific for both *E. coli* O9 and *S. typhimurium* on the cell surface, while the *rfc* mutant was expressed only O9-specific LPS. *E. coli* O-antigen was also synthesized in the *S. typhimurium* *rfb* mutant but not in the *rfe* mutant. Sugiyama et al., *supra*. This group  
15 concluded that gene products of the *S. typhimurium* *rfa* and *E. coli* O9 *rfb* loci can cooperate to synthesize *E. coli* O9-antigen on the R-core of *S. typhimurium*. However, this group did not report whether any of these recombinant *S. typhimurium* constructs could grow within an animal host or generate a protective host immune response against wild-type *E. coli* O9 or *S. typhimurium*. Accordingly, a need exists  
20 for a bivalent vaccine to control *Salmonella* and *E. coli* infection in poultry. Such a vaccine would simultaneously benefit the public health and reduce the costs of poultry production.

#### Summary of the Invention

25 In one embodiment the present invention is directed to a vaccine that protects birds against infection by an avian pathogenic gram-negative (AP<sub>G-N</sub>) microbe. The vaccine comprises live cells of a recombinant *Salmonella* strain expressing O-antigen of the AP<sub>G-N</sub> microbe due to integration into the *Salmonella* chromosome of the *rfb* gene cluster and the *rfc* gene of the AP<sub>G-N</sub> (hereinafter used interchangeably with  
30 AP<sub>G-N</sub> *rfb/rfc* gene cluster). The recombinant *Salmonella* strain, which is an attenuated mutant of a virulent *Salmonella* strain, does not express O-antigen of the virulent *Salmonella* strain due to a mutation in the *Salmonella* *rfb* gene cluster and/or

in the *Salmonella rfc* gene. In a preferred embodiment, the AP<sub>G-N</sub> microbe is an APEC strain and the AP<sub>G-N</sub> *rfb/rfc* gene cluster is an APEC *rfb/rfc* gene cluster.

This recombinant *Salmonella* strain has other features that make it particularly useful as a vaccine in poultry and other birds. First, the vaccine can be formulated for oral administration and oral vaccines are known to stimulate the gut associated lymphoid tissue (GALT), including mucosal, humoral and cellular immune responses. Oral, live vaccines also cost less to produce and are easier to administer in the field than injectable vaccines. Second, the lack of expression of LPS O-antigen specific for the carrier strain avoids any interference that the carrier LPS O-antigen might have on expression of the AP<sub>G-N</sub> O-antigen or its recognition by the vaccine recipient's immune system. Third, the recombinant *Salmonella* strain can protect against both AP<sub>G-N</sub> microbes and the parental *Salmonella* strain because it expresses other cell-surface antigens of the parental *Salmonella* strain. In addition, for vaccines expressing O-antigen from an APEC strain, use of *Salmonella* rather than *E. coli* as the carrier bacteria should provide a more vigorous immune response against the APEC O-antigen because while *S. enterica* subspecies persist in the spleen and bursa of Fabricius, *E. coli* does not effectively invade these lymphoid tissues or is quickly killed even if occasionally successful in entering them.

In some embodiments, the recombinant *Salmonella* strain used in the vaccine also contains a recombinant polynucleotide encoding a desired gene product. A preferred gene product is an antigen from an avian pathogenic gram-positive (AP<sub>G-P</sub>) microbe or from a eukaryotic avian pathogen.

In another embodiment, the invention provides a multivalent vaccine for immunizing birds against at least two avian pathogenic gram-negative (AP<sub>G-N</sub>) microbes which comprises live cells of a recombinant *Salmonella* strain expressing an O-antigen of each of the AP<sub>G-N</sub> microbes, the recombinant *Salmonella* strain having an *rfb/rfc* gene cluster of each of the AP<sub>G-N</sub> microbes integrated into the *Salmonella* chromosome and having a mutation in the *Salmonella rfb* gene cluster or in the *Salmonella rfc* gene which inactivates expression of *Salmonella* O-antigen, wherein the recombinant *Salmonella* strain is an attenuated mutant of a virulent *Salmonella* strain. In a preferred embodiment, one or both of the AP<sub>G-N</sub> microbes is an APEC strain.

In yet another embodiment, the invention provides a multivalent vaccine for immunizing a bird against at least two AP<sub>G-N</sub> microbes which comprises a mixture of live cells of first and second recombinant *Salmonella* strains, the first recombinant *Salmonella* strain having an *rfb/rfc* gene cluster of a first AP<sub>G-N</sub> microbe integrated  
5 into the *Salmonella* chromosome and expressing O-antigen of the first AP<sub>G-N</sub> microbe and the second recombinant *Salmonella* strain having an *rfb/rfc* gene cluster of a second AP<sub>G-N</sub> microbe integrated into the *Salmonella* chromosome and expressing O-antigen of the second AP<sub>G-N</sub> microbe, wherein each of the first and second recombinant *Salmonella* strains has a mutation in the *Salmonella rfb* gene cluster or in  
10 the *Salmonella rfc* gene which inactivates expression of *Salmonella* O-antigen, and wherein each of the first and second recombinant *Salmonella* strains is an attenuated mutant of a virulent *Salmonella* strain. In a preferred embodiment, one or both of the recombinant *Salmonella* strains in the multivalent vaccine express O-antigen of an APEC strain.

15 The present invention in other embodiments is directed to methods for immunizing birds against infection by AP<sub>G-N</sub> microbes. These methods include a method for immunizing a bird against an AP<sub>G-N</sub> microbe which comprises administering to the bird an immunologically effective amount of a vaccine comprising live cells of a recombinant *Salmonella* strain expressing O-antigen of the  
20 AP<sub>G-N</sub> microbe, the recombinant *Salmonella* strain having an *rfb/rfc* gene cluster of the AP<sub>G-N</sub> microbe stably integrated into the *Salmonella* chromosome and having a mutation in the *Salmonella rfb* gene cluster or in the *Salmonella rfc* gene which inactivates expression of *Salmonella* O-antigen, wherein the recombinant *Salmonella* strain is an attenuated mutant of a virulent *Salmonella* strain. The methods also  
25 include a method for simultaneously immunizing a bird against more than one AP<sub>G-N</sub> microbes which comprises administering to the bird an immunologically effective amount of a multivalent vaccine as described above. Also included is a method for simultaneously immunizing a bird against an AP<sub>G-N</sub> microbe and the carrier *Salmonella* species which comprises administering to the bird an immunologically  
30 effective amount of any of the vaccines as described above.

In still another embodiment, the invention provides a method of making a vaccine for immunizing a bird against an AP<sub>G-N</sub> microbe strain. The method comprises the steps of selecting a *Salmonella* strain capable of colonizing the bird,

integrating into the *Salmonella* chromosome an *rfb/rfc* gene cluster from the AP<sub>G-N</sub> microbe, introducing a mutation into the *Salmonella rfb* gene cluster and/or into the *Salmonella rfc* gene, and isolating recombinant *Salmonella* bacteria which express O-antigen characteristic of the AP<sub>G-N</sub> microbe but which do not express *Salmonella* O-antigen. In one embodiment the selected *Salmonella* strain is an attenuated mutant of a virulent *Salmonella* strain. In another embodiment, the selected *Salmonella* strain is a virulent *Salmonella* strain and the method further comprises the step of introducing into the virulent *Salmonella* strain an attenuating mutation into a *Salmonella* virulence gene and isolating mutants having attenuated virulence as compared to the virulent *Salmonella* strain.

Among the several advantages achieved by the present invention, therefore, is the provision of live, recombinant *Salmonella* vaccines capable of protecting birds and particularly poultry against infection by APEC strains and other AP<sub>G-N</sub> microbes, and methods for making such vaccines, the provision of multivalent vaccines useful for simultaneously protecting birds against infection by two or more AP<sub>G-N</sub> microbes, the provision of methods for immunizing birds against APEC strains and other AP<sub>G-N</sub> microbes, and the provision of a method for immunizing birds against both APEC and *Salmonella* bacteria.

#### Brief Description of the Drawings

Figure 1 is a genetic map of the suicide cosmid pMEG219 showing the following features: a *pir*-dependent origin of replication (R6K ori); the tetracycline resistance genes from Tn10 (*tetR*, *tetA*); a mobilization fragment from plasmid RK2 (*mob*), the kanamycin resistance gene (*Kan*) and double *cos* sites (*cos*) from pCOS2EMBL; and the *S. typhimurium*  $\Delta$ *cydA*-27 allele; with the location of *NotI* and *PvuII* restriction sites described in the text indicated;

Figure 2 shows digitized images of western blots of LPS isolated from the indicated bacteria strain probed with (Fig. 2A) anti-*S. typhimurium* group B LPS antibody or (Fig. 2B) anti-O78 LPS antibody;

Figure 3 shows digitized images of western blots of LPS isolated from the indicated bacteria strain probed with (Fig. 3A) anti-*S. typhimurium* group B LPS antibody or (Fig. 3B) anti-O78 LPS antibody, with a molecular weight marker included in the gel for the blot shown in Fig. 3A;



Figure 4 is a genetic map of pMEG287 showing the location of genes discussed in the text; and

Figure 5 is a genetic map of pMEG055 showing the location of genes discussed in the text.

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#### Detailed Description of the Invention

The present invention is based on the discovery that *Salmonella enterica* subspecies, in particular *S. typhimurium*, can be engineered to express O antigen of AP<sub>G-N</sub> microbes, in particular of O78 and O1 APEC strains, in an immunogenic form on the cell surface without such expression interfering with *in vivo* growth or colonization of the poultry GALT by the *Salmonella* carrier. In addition, the inventor herein has discovered that oral administration of such recombinant *Salmonella* strains to chickens results in a significant reduction in the ability of virulent APEC strains to infect and cause disease in the vaccinated chickens.

Thus, one embodiment of the invention is a vaccine for immunization of birds against an avian pathogenic gram-negative microbe (AP<sub>G-N</sub>) comprising live cells of a recombinant *Salmonella* strain expressing O-antigen of the AP<sub>G-N</sub> strain. The AP<sub>G-N</sub> O-antigen is expressed in an immunogenic form, meaning that the AP<sub>G-N</sub> O-antigen moiety is part of a complete LPS O-antigen molecule in which the AP<sub>G-N</sub> O-antigen is attached to an LPS core moiety and, upon administration to a bird, is capable of generating antibodies that react with LPS O-antigen of the wild-type AP<sub>G-N</sub> microbe. Typically, the LPS core moiety is synthesized by the recombinant *Salmonella* carrier, but in some embodiments the LPS core may be from the same AP<sub>G-N</sub> microbe as the O-antigen or from a different AP<sub>G-N</sub> microbe.

It is contemplated that the vaccine can be used to immunize all types of birds, including chickens, turkeys, ducks, geese, pheasants and other domesticated birds categorized as poultry, as well as non-domesticated birds such as wild turkeys and exotic species such as parrots, parakeets, etc., against any AP<sub>G-N</sub> microbe now known or subsequently determined to be pathogenic in birds. Such AP<sub>G-N</sub> microbes include but are not limited to: (1) APEC strains such as *E. coli* serotypes O1, O2, O3, O6, O8, O15, O18, O35, O71, O74, O78, O87, O88, O95, O103 and O109; (2) avian pathogenic *Salmonella* strains, e.g., group C and group D strains; and (3) species of the following genera: *Campylobacter*, *Bacteroides*, *Bordetella*, *Haemophilus*,

*Pasteurella*, *Francisella*, *Actinobacillus*, *Klebisella*, *Moraxella*, *Pseudomonas*, *Proteus*, and *Ornithobacterium*. Preferably, the recombinant *Salmonella* strain expresses O-antigen of an APEC strain. More preferably, the APEC O-antigen is characteristic of the O1, O2, O35 or O78 serotypes and most preferably the O-antigen is O1 LPS, O2 LPS or O78 LPS.

The recombinant *Salmonella* strain used in the vaccine is prepared by integrating the *rfb/rfc* gene cluster from a desired AP<sub>G-N</sub> microbe into the chromosome of a suitable *Salmonella* strain. *Salmonella* strains for use as the carrier bacteria may be derived from any *Salmonella* species that is capable of colonizing birds, preferably poultry. Such species include but are not limited to *S. typhimurium*, *S. enteritidis*, *S. gallinarum*, *S. pullorum*, *S. arizona*, *S. heidelberg*, *S. anatum*, *S. hadar*, *S. agana*, *S. montevideo*, *S. kentucky*, *S. infantis*, *S. schwarzengrund*, *S. saintpaul*, *S. brandenburg*, *S. instambul*, *S. cubana*, *S. bredeney*, *S. braenderup*, *S. livingstone*, *S. berta*, *S. californica*, *S. senftenberg*, and *S. mbandaka*. As used herein, "colonizing" means the *Salmonella* species is able to attach to, invade and persist in one or more of the following tissues in the vaccinated bird: lung, spleen, liver, bursa of Fabricius, and ceca. Preferably, the recombinant *Salmonella* strain is derived from *S. typhimurium*, *S. gallinarum*, *S. pullorum*, or *S. enteritidis*. Most preferably, the AP<sub>G-N</sub> *rfb/rfc* gene cluster is inserted into a strain of *S. typhimurium*.

As used herein, an AP<sub>G-N</sub> *rfb/rfc* gene cluster contains all the genes necessary for synthesis of O-antigen characteristic of that AP<sub>G-N</sub> microbe, including all *rfb* genes whose products are required for biosynthesis of the monomer sugar unit, as well as the *rfc* gene, which encodes the O-antigen polymerase needed for polymerization of the sugar units. The terms *rfb* genes and *rfc* gene are intended to mean those genes of any AP<sub>G-N</sub> microbe identified as encoding gene products having the same function in O-antigen biosynthesis as the products of an *E. coli* *rfb/rfc* gene cluster. In most *E. coli* and *Salmonella* serotypes, the *rfc* gene is tightly linked to the *rfb* gene cluster, which in *E. coli* is about 10kb in length and located near 45 minutes on the *E. coli* genetic map. However, in some *E. coli* strains, as in *S. typhimurium*, the *rfc* gene is not linked to the *rfb* gene cluster and can be found a significant distance away. For example, in *S. typhimurium*, the *rfb* gene cluster, which is about 21 kb, extends from 44.9-45.3 centisomes on the genetic map of *S. typhimurium* while the *rfc* gene is located at 35.7 centisomes.

A functionally complete AP<sub>G-N</sub> *rfb/rfc* gene cluster can readily be cloned using routine cosmid cloning methodology, as previously described for cloning bacteria *rfb/rfc* gene clusters. See, for example, Viret et al., EP 0 564 689 B1; Neal et al., *FEMS Microbiol. Lett.* 82:345-352, 1991; Haraguchi et al., *Microb. Pathog.* 6:123-132, 1989; *Microb. Pathog.* 10:351-361, 1991; Heuzenroeder et al., *Mol. Microbiol.* 3:295-302, 1989; Bastin et al., *Mol. Microbiol.* 5:2223-2231, 1991; and Valvano et al., *Infect. Immun.* 57:937-943, 1989. General cosmid cloning techniques are also described in detail in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, 1989. In brief, a cosmid library of chromosomal DNA from the desired AP<sub>G-N</sub> microbe is prepared in the *E. coli* K-12 strain, which does not produce O-antigen due to mutations in the *rfb* region. The cosmid library is plated and isolated colonies are screened for expression of the AP<sub>G-N</sub> O-antigen using antisera specific for the O-antigen of the desired AP<sub>G-N</sub> strain. The AP<sub>G-N</sub> chromosomal DNA fragment can be isolated from positive cosmid clones and further characterized using standard techniques, such as restriction mapping and subcloning, to determine the minimal amount of AP<sub>G-N</sub> DNA required to direct synthesis of AP<sub>G-N</sub> O-antigen. This minimal amount is herein referred to as the *rfb/rfc* gene cluster. For those AP<sub>G-N</sub> microbes where the *rfc* gene is located a considerable distance from the *rfb* gene cluster, a single AP<sub>G-N</sub> chromosomal fragment containing a functional *rfb/rfc* gene cluster may not be represented in the cosmid library. In such cases, additional routine cloning and screening experiments may need to be performed to clone the *rfc* gene and construct a recombinant fragment containing both the *rfb* genes and the *rfc* gene. The *rfb/rfc* gene cluster is then transfected as a cosmid or plasmid into the selected *Salmonella* strain and the LPS produced by the resulting recombinant is analyzed by methods known in the art and those briefly described below.

It has been reported that the *Salmonella* LPS core moiety can not accept heterologous O-antigen of some bacteria, resulting in unbound O-antigen, which is probably less immunogenic than complete LPS molecules carrying the heterologous O-antigen covalently attached to the core moiety of the carrier bacteria (EP 0 564 689 B1; Morona et al., U.S. Patent No. 5,110,588). Recombinant strains expressing complete LPS O-antigen can be distinguished from those expressing unbound O-antigen by SDS-PAGE analysis of LPS extracted from late exponential or stationary

cultures using a rapid small-scale mini-prep method (Hitchcock and Brown, *J. Bacteriol.* 154:269-277, 1983). The separated LPS molecules can be detected in the gel by silver staining or by immunoblot analysis using an antibody specific for the O-antigen. Complete LPS O-antigen (also referred to herein as smooth, or S-type LPS) will produce a ladder of high molecular weight bands detectable by either silver staining or immunoblot analysis, whereas unbound O-antigen does not produce a ladder and will be detected as a smear by silver staining or probing with antibody. In such cases, the *Salmonella rfa* locus, which directs synthesis of the LPS core, can be swapped with an *rfa* locus of another bacteria, such as *E. coli* K-12 or the AP<sub>G-N</sub> microbe, to derive a combination of *rfa* and AP<sub>G-N</sub> *rfb/rfc* gene clusters that will direct synthesis of AP<sub>G-N</sub> O-antigen in an immunogenic form.

To assure stable expression of the heterologous antigen and eliminate the need for use of antibiotics to maintain an extrachromosomal element in the *Salmonella* strain, the AP<sub>G-N</sub> *rfb/rfc* gene cluster is integrated into the *Salmonella* chromosome. This can be accomplished by any known methodology, preferably by a defined deletion/insertion mutation into a virulence gene to produce an attenuated mutant as described below. Alternatively, AP<sub>G-N</sub> *rfb/rfc* gene cluster can be integrated in a manner that does not attenuate the recombinant *Salmonella* strains.

The recombinant *Salmonella* strain also contains a mutation in the *Salmonella rfc* gene which inactivates expression of the *Salmonella* O-antigen polymerase. *Salmonella* mutants defective in the O-antigen polymerase produce LPS molecules termed semirough (SR LPS), which have at most one O-antigenic sugar unit attached to any given core unit instead of the usual 30-40 sugar units seen in Rfc<sup>+</sup> strains. These SR LPS molecules are less likely to interfere with the ability of the much longer APEC LPS O-antigen to stimulate a protective immune response. The recombinant *Salmonella* strain can be prepared from a strain already containing an *rfc* mutation or the *rfc* mutation can be introduced at the same time as, or after, integration of the APEC *rfb/rfc* gene clusters into the *Salmonella* chromosome.

*Salmonella rfc* mutants can be created using routine techniques known in the art. For example, random transposon insertions can be made in the chromosome of the desired *Salmonella* strain (see, e.g., Curtiss, U.S. Patent 5,672,345) and mutants having a Rfc<sup>-</sup> phenotype (i.e., expression of SR LPS) isolated (Collins et al., *J.*

*Bacteriol.*:173:2521-2529, 1991). Alternatively, a deletion mutation can be introduced into the *Salmonella rfc* gene using recombinant DNA techniques such as those described in the Examples herein. In brief, the *rfc* gene of *S. typhimurium* has been cloned and sequenced and is believed to be conserved among *Salmonella* strains of serogroups A, B and D1 (Collins et al., *supra*). Based on this information the *rfc* gene from a *Salmonella* strain belonging to one of these serogroups can be cloned and used to construct a suicide plasmid containing a deletion in the cloned *rfc* gene. Introduction of this plasmid into an *rfc*<sup>+</sup> *Salmonella* strain will lead to homologous recombination between the chromosomal *rfc* gene and the  $\Delta rfc$  plasmid. The transformed cells are then cultured on media lacking selection for the plasmid and the isolates screened for the Rfc<sup>-</sup> phenotype.

*Salmonella* mutants can be tested for an Rfc<sup>-</sup> phenotype by SDS-PAGE analysis of LPS isolated from the bacteria and detection of a high proportion of SR LPS with an O-sugar unit specific for the *Salmonella* strain. Another procedure that can be used to screen *Salmonella* mutants for the Rfc<sup>-</sup> phenotype is by a bacteriophage sensitivity assay, using a phage specific for s-type LPS. For example, *S. typhimurium* and other *Salmonella* group B strains having an Rfc<sup>+</sup> phenotype (i.e., express s-type LPS) are lysed by P22 whereas Rfc<sup>-</sup> mutants of these strains are resistant to P22-mediated lysis as P22 does not recognize the SR LPS expressed by these mutant strains.

It is also contemplated that a recombinant *Salmonella* strain expressing AP<sub>G-N</sub> O-antigen from more than one AP<sub>G-N</sub> microbe can be constructed using techniques similar to those described herein for preparing strains expressing a single AP<sub>G-N</sub> O-antigen.

Because the vaccine comprises live cells of the recombinant *Salmonella* strain, it is possible that live, recombinant *Salmonella* bacteria could be transmitted to human consumers of poultry food products such as eggs and meat. In addition, *S. enterica* species can cause disease in very young birds. Thus, an important feature of the invention is that the recombinant *Salmonella* strain is an attenuated mutant of a virulent strain of an *S. enterica* species. As used herein, an attenuated mutant means that the recombinant *Salmonella* strain has the ability to colonize and replicate in a

vaccinated bird but which is substantially incapable of causing the disease symptoms associated with infection of the particular avian species being treated or infection of humans by its virulent counterpart. By the term "substantially incapable of causing disease symptoms" is meant the attenuated mutant either produces no disease  
5 symptoms or produces less severe and/or a fewer number of such symptoms. However, an attenuated mutant strain is not necessarily incapable of causing some effect on normal physiological function in the vaccine recipient or a human and may be a pathogen in avian species other than the intended vaccine recipient as well as in other nonhuman hosts.

10           The recombinant *Salmonella* strain can be derived from naturally occurring avirulent mutants of virulent strains of *S. enterica* species, or by introducing attenuating mutations into wild-type, virulent strains using well-known techniques. Attenuating mutations can be in biosynthetic genes, regulatory genes and/or genes involved in virulence. (See Doggett and Brown, in *Mucosal Vaccines*, Kiyono et al.,  
15 eds., Academic Press, San Diego, 1996, pp. 105-118). Examples of genes whose mutation will lead to attenuation include, but are not limited to a mutation in a *pab* gene, a *pur* gene, an *aro* gene, *asd*, a *dap* gene, *nadA*, *pncB*, *galE*, *pmi*, *fur*, *rpsL*, *ompR*, *htrA*, *hemA*, *cdt*, *cya*, *crp*, *phoP*, *phoQ*, *rfc*, *poxA*, *galU* and combinations thereof. Mutations can be insertions, partial or complete deletions or the like so long  
20 as expression of the gene is diminished and virulence is decreased. The skilled artisan will readily appreciate that any suitable gene mutation can be used in the present invention so long as the mutation of that gene renders the microorganism attenuated. Preferably, the carrier microbes of the invention have at least two mutations, each of which act to attenuate the microbe and which, in combination, significantly increase  
25 the probability that the microbe will not revert to wild-type virulence.

Methods are known in the art that can be used to generate mutations to produce the attenuated recombinant *Salmonella* strain of the present invention. For example, the transposon, Tn10, can be used to produce chromosomal deletions in a wide variety of bacteria, including *Salmonella* (Kleckner et al., *J. Mol. Biol.* 116:125-  
30 159, 1977; EPO Pub. No. 315,682; U.S. Patent No. 5,387,744).

Recently, new methods have become available for producing specific deletions in genes. These methods involve initially selecting a gene in which the deletion is to be generated. In one approach the gene can be selected from a genomic

library obtained commercially or constructed using methods well known in the art (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., 1989, Cold Spring Harbor Press, Cold Spring Harbor, NY). Clones containing the gene are isolated from the genomic library by complementation of a strain which contains a  
5 mutation in the same gene. Alternatively, when the DNA sequence of the gene is known, selected primers for the polymerase chain reaction method (PCR) can amplify the gene, often with some flanking sequence, from a sample of bacteria or from purified genomic DNA and the PCR product can be inserted into a cloning vector.

A specific deletion in the selected gene can be generated by either of two  
10 general methods. The first method generates a mutation in a gene isolated from a population of clones contained in a genomic DNA library using restriction enzymes and the second method generates the mutation in a gene of known sequence using PCR. Using the first method, the position of the gene on a vector is identified using transposon tagging and a restriction map of the recombinant DNA in the vector is  
15 generated. Information derived from the transposon tagging allows all or a portion of a gene to be excised from the vector using the known restriction enzyme sites. The second method, which is based upon PCR methodology, can be used when the DNA sequence of the gene is known. According to this method, divergent PCR primers amplify the upstream and downstream regions flanking a specified segment of DNA  
20 to be deleted from the gene and generate a PCR product consisting of the cloning vector and upstream and downstream flanking nucleotide sequences (Innes et al. Eds., *PCR Protocols*, 1990, Academic Press, New York). In a variation of this method, PCR products are produced representing portions of the gene or flanking sequence, which are then joined together in a cloning vector.

25 The DNA containing the mutant gene can be introduced into the bacterial host by transformation using chemical means or electroporation, by recombinant phage infection, or by conjugation. In preferred embodiments, the mutant gene is introduced into the chromosomes of the bacteria which can be accomplished using any of a number of methods well known in the art such as, for example, methods  
30 using temperature-sensitive replicons (Hamilton et al., *J. Bacteriol.* 171:4617-4622, 1989), linear transformation of *recBC* mutants (Jasin et al., *J. Bacteriol.* 159:783-786, 1984), or host restricted replicons known as suicide vectors (Miller et al., *J. Bacteriol.* 170:2575-2583, 1988). The particular method used is coupled with an appropriate

counter selection method such as, for example, fusaric acid resistance or sucrose resistance followed by subsequent screening for clones containing the mutant allele based upon phenotypic characteristics or by using PCR, nucleic acid hybridization, or an immunological method.

5           The recombinant *Salmonella* strain used in the present invention can also be used to deliver a desired gene product to the vaccinated bird. The term "gene product" as used herein refers to any biological product or products produced as a result of the biochemical reactions that occur under the control of a gene. The gene product can be, for example, an RNA molecule, a peptide, a protein, or a product  
10       produced under the control of an enzyme or other molecule that is the initial product of the gene, i.e., a metabolic product. For example, a gene can first control the synthesis of an RNA molecule which is translated by the action of ribosomes into an enzyme which controls the formation of glycans in the environment external to the original cell in which the gene was found. The RNA molecule, the enzyme, and the  
15       glycan are all gene products as the term is used here. Examples of desired gene products include but are not limited to antigens, various host cell proteins, pharmacologically active products, toxins, and apoptosis-modulating agents.

          An antigen or immunogen is intended to mean a molecule containing one or more epitopes that can stimulate a host immune system to make a secretory,  
20       humoral and/or cellular immune response specific to that antigen. An epitope can be a site on an antigen to which an antibody specific to that site binds. For protein antigens, an epitope could comprise 3 amino acids in a spatial conformation which is unique to the epitope; generally, an epitope will consist of at least 6 consecutive amino acids, or more usually, at least 10-12 consecutive amino acids of the antigen.  
25       The term "epitope" is intended to be interchangeable with the term "antigenic determinant" as used herein. The term "epitope" is also intended to include T-helper cell epitopes in which an antigenic determinant is recognized by T-helper cells through association with major histocompatibility complex class II molecules. In addition, the term epitope includes any antigen, epitope or antigenic determinant  
30       which is recognized by cytotoxic T cells when presented by a MHC class I molecule on the surface of an antigen presenting cell. A cytotoxic T cell epitope can comprise an amino acid sequence of between about 6 to about 11 consecutive amino acids, and preferably comprises a sequence of 8 or 9 consecutive amino acids.



If the desired gene product is an antigen from a bacterial, fungal, parasitic or viral disease agent, the recombinant *Salmonella* strain can be used to vaccinate birds against diseases caused by such agents at the same time as vaccinating against an AP<sub>G-N</sub> microbe. For example, the recombinant *Salmonella* strain could be used to  
5 deliver an antigen from an avian pathogenic microbe that does not express O-antigen such as gram-positive (AP<sub>G-P</sub>) bacteria. Such microbes include but are not limited to species of *Mycoplasma*, *Listeria*, *Borrelia*, *Chlamydia*, *Clostridia*, *Corynebacteria*, *Coxiella*, *Eysipelothrix*, *Flavobacteria*, *Staphylococcus*, and *Streptococcus*. Examples of fungal and parasitic avian pathogens known to infect poultry are species of  
10 *Amoebotaenia*, *Aproctella*, *Ascaridia*, *Aspergillus*, *Candida*, *Capillaria*, *Cryptosporidium*, *Cyathostroma*, *Dispharynx*, *Eimeria*, *Fimbriaria*, *Gongylonemia*, *Heterakis*, *Histomonas*, *Oxyspirura*, *Plasmodium*, *Raillietina*, *Strongyloides*, *Subulura*, *Syngamus*, *Tetrameres*, and *Trichostrongylus*. Viruses known to infect poultry include adenoviruses (e.g., hemorrhagic enteritis virus), astroviruses,  
15 coronaviruses (e.g., Infectious bronchitis virus), paramyxoviruses (e.g., Newcastle disease virus), picornaviruses (e.g., avian encephalomyelitis virus), pox viruses, retroviruses (e.g., avian leukosis/sarcoma viruses), reoviruses, and rotaviruses. Preferred gene products for use as antigens are polysaccharides and proteins, including glycoproteins and lipoproteins. Antigen-encoding genes from these  
20 prokaryotic and eukaryotic organisms can be cloned and expressed in the recombinant *Salmonella* strain using standard techniques.

In other embodiments, the desired gene product directs the expression of a gamete-specific antigen which is capable of eliciting an immune response that confers an antifertility effect upon the immunized animal (See, U.S. Patent 5,656,488).

25 As used herein, vaccine means an agent used to stimulate the immune system of an animal so that protection is provided against an antigen not recognized as a self-antigen by the immune system. Immunization refers to the process of inducing a continuing high level of antibody and/or cellular immune response in which T-lymphocytes can either kill the pathogen and/or activate other cells (e.g.,  
30 phagocytes) to do so in the immunized animal, which is directed against a pathogen or antigen to which the animal has been previously exposed. In this application the phrase "immune system" is intended to refer to the anatomical features and mechanisms by which an avian species produces antibodies against an antigenic

material which invades the cells of the vertebrate or the extra-cellular fluid of the individual and is also intended to include cellular immune responses.

In the case of antibody production, the antibody so produced can belong to any of the immunological classes, such as immunoglobulins, A, D, E, G or M. Of particular interest are vaccines which stimulate production of immunoglobulin A (IgA) since this is the principle immunoglobulin produced by the secretory system of avian species, although vaccines of the invention are not limited to those which stimulate IgA production. For example, vaccines of the nature described herein are likely to produce a broad range of other immune responses in addition to IgA formation, for example cellular and humoral immunity. Immune responses to antigens are well studied and widely reported.

The avirulent microbes of this invention can also be used as vectors for synthesis of other proteins, including immunoregulatory molecules made by avian species and pharmacologically active products that might stimulate or suppress various physiological functions (i.e., growth rate, fat or protein content, etc.).

The desired gene product is encoded by a recombinant polynucleotide. The term "recombinant polynucleotide" is defined herein to refer to the result of laboratory manipulations which results in the introduction into the *Salmonella* strain of a promoter operably linked to a gene from various endogenous and/or exogenous sources. The promoter is one that is functional in the *Salmonella* strain to produce expression of the gene. The gene can be of chromosomal, plasmid, or viral origin. A gene as used herein is any biological unit of heredity capable of producing a desired gene product. It is not, however, necessary that the gene be a complete gene as is present in the parent organism and capable of producing or regulating the production of a macromolecule such as for example, a functioning polypeptide. The recombinant polynucleotide may, thus, encode all or part of an antigenic product. A gene can also refer to a polynucleotide having a sequence mutated from the naturally-occurring sequence found in the parent organism. The recombinant polynucleotide can also refer to a long section of DNA coding for several gene products, one or all of which can be antigenic or part of a biosynthetic pathway that leads to the desired gene product. For example, such a long section of DNA could encode 5 to 15 proteins necessary for the synthesis of fimbrial antigens (fimbriae), which mediate adhesion of pathogens to host cells (Bäumler et al., *supra*). The induction of an immune response

against fimbriae can provide protection against the pathogen. It is to be understood that the term gene as used herein further includes DNA molecules lacking introns such as, for example, is the case for cDNA molecules, so long as the DNA sequence encodes the desired gene product. The recombinant polynucleotide encoding the  
5 desired gene product may include DNA sequences other than the promoter such as termination sequences and other regulators of prokaryotic gene expression.

The recombinant polynucleotide encoding a desired gene product can be transferred into the *Salmonella* strain in the form of a plasmid, phage or cosmid vector by various means such as conjugation, electroporation, or transformation (uptake of  
10 naked DNA from the external environment, which can be artificially induced by the presence of various chemical agents, such as calcium ions). Other methods such as transduction are also suitable, wherein the recombinant DNA in the form of a transducing phage or cosmid vector is packaged within a phage. Once the recombinant polynucleotide is in the carrier *Salmonella*, it may continue to exist as a  
15 separate autonomous replicon or it may insert into the *Salmonella* chromosome and be reproduced along with the chromosome during cell division.

Preferably, the recombinant polynucleotide is incorporated into a "balanced-lethal" system which selects for microorganisms containing and capable of expressing the desired gene product by linking the survival of the microorganism to  
20 the continued presence of the recombinant polynucleotide. "Balanced-lethal" mutants of this type are characterized by a lack of a functioning native chromosomal gene encoding an enzyme which is essential for cell survival, preferably an enzyme which catalyzes a step in the biosynthesis of diaminopimelic acid (DAP) and even more preferably a gene encoding beta aspartate semialdehyde dehydrogenase (Asd). DAP  
25 pathway enzymes and Asd are required for cell wall synthesis. "Balanced-lethal" mutants also contain a recombinant gene which can serve to complement the non-functioning chromosomal gene and this complementing recombinant gene is structurally linked to the recombinant polynucleotide encoding the desired gene product. Loss of the complementing recombinant gene causes the cells to die by lysis  
30 when the cells are in an environment where DAP is lacking. This strategy is especially useful since DAP is not synthesized by eukaryotes and, therefore, is not present in infected avian tissues. Methods of preparing these types of "balanced lethal" microbes are disclosed in U.S. Patent No. 5,672,345.

Administration of the vaccine to a bird may be by any known or standard technique, including mucosal or intramuscular injection. Preferred administration methods include oral ingestion or broncho-nasal-ocular spraying. These methods allow the recombinant *Salmonella* to easily reach the gut-associated lymphoid tissue (GALT) or bronchus-associated lymphoid tissue (BALT) and induce antibody formation and cell mediated immunity. A particularly preferred administration method is to vaccinate newborn birds, e.g., on the day of hatch, by coarse spray.

After growth and harvesting of the recombinant *Salmonella* strain, the bacterial cells may be lyophilized, particularly if they are to be mixed in foodstuffs. The vaccine comprised of the recombinant *Salmonella* bacteria may be prepared using any excipient that is pharmaceutically acceptable for the type of bird being immunized. For example, if the vaccine is to be administered in solid form, the cells may be coated with and/or encapsulated in a material that is non-toxic to the inoculated bird and compatible with the bacteria. Solid carriers such as talc or sucrose may also be used. If the administration is to be in liquid form, the cells may be suspended in a suitable liquid carrier, including for example, skim milk, normal saline and/or other non-toxic salts at or near physiological concentrations, and other suitable liquid carriers known to those of skill in the art. Where desirable, adjuvants may also be added to enhance the antigenicity. When the vaccine is intended for administration as a spray, the recombinant *Salmonella* cells may be suspended in a suitable buffer such as BSG (buffered saline with gelatin, Curtiss III, R., *J. Bacteriol.* 89:28-40, 1965).

The dosage required will vary depending on the quantity and antigenicity of the APEC LPS O-antigen expressed by the recombinant *Salmonella* strain as well as the type, size and age of bird to be vaccinated. For example, a lower dosage may be required for vaccinating newborn birds than the dosage suitable for vaccinating older birds. Routine experimentation will easily establish the required amount. Generally, the dosage will be in concentrations ranging from  $10^5$  to  $10^9$  live cells per bird. A preferred dosage for spray vaccination of newborn chickens with vaccines comprising live recombinant *S. typhimurium* cells is about  $10^6$  to  $10^8$  live cells/bird and a particularly preferred dosage is about  $5 \times 10^7$  live cells/bird.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent

to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

5

## Example 1

This example illustrates the construction of an attenuated recombinant *S. typhimurium* strain coexpressing *S. typhimurium* group B LPS and *E. coli* O78 LPS.

Attenuation strategy. *S. typhimurium* strains carrying deletions in the *cya* and *crp* genes have been shown to be attenuated and immunogenic in chickens (Hassan et al., *Res. Microbiol.* 141:839-850, 1990; Porter et al., *Avian Dis.* 37:265-273, 1993) and, in addition, to elicit a protective immune response against wild-type *S. typhimurium* challenge in this species (Hassan et al., *supra*, Hassan et al., *Infect. Immun.* 62:5519-5527, 1994). Biochemically, deletions in either *cya* or *crp* result in an inability to ferment a variety of sugars, including maltose (Botsford et al., *Microbiol. Rev.* 1992). Therefore,  $\Delta cya \Delta crp$  *S. typhimurium* vaccine strains form white colonies on MacConkey maltose media, while wild-type *S. typhimurium* strains form red colonies, thus allowing a simple method for discrimination between the vaccine strain and wild-type *S. typhimurium*. Based upon these earlier findings, a  $\Delta cya \Delta crp$  *S. typhimurium* was chosen as the carrier strain.

20

Insertion of the O78 *rfb/rfc* gene cluster into the *Salmonella* chromosome.

To facilitate the insertion of the O78 *rfb* into the *S. typhimurium* chromosome and produce an attenuating deletion mutation in the *Salmonella cya* gene in a single step, a *pir*-dependent cosmid vector, pMEG219, was constructed. As illustrated in Fig. 1 and in Table 1 below, pMEG219 has the following features: a *pir*-dependent origin of replication, the tetracycline resistance genes from Tn10, a mobilization fragment from plasmid RK2, the kanamycin resistance gene and double *cos* sites from pCOS2EMBL, and the cloned *S. typhimurium*  $\Delta cya$ -27 allele. Because the origin is *pir*-dependent, this cosmid can replicate only in special *E. coli* strains that have been engineered to supply the Pir protein (Kolter et al., *Cell* 15:1199-1208, 1978), but not in wild-type *S. typhimurium* strains. The *cya*-27 allele contains a defined deletion of *cya* which consists of a 327 bp deletion of the upstream region of *cya* including the first 13 codons, as well as 476 bp of flanking sequence upstream and 1,826 bp of

30

flanking sequence downstream of the deletion. In addition, a *NotI* site was engineered at the point of the deletion to facilitate the insertion of DNA in the *cya* gene (P. Sundaram, personal communication).

**Table 1. Bacterial strains and plasmids**

5	Strain	Relevant genotype	Source or reference
	<i>E. coli</i>		
10	$\chi$ 7122	avian pathogenic strain 078:K80:H9, nal <sup>r</sup>	Provence et al., <i>Infect. Immun.</i> 60:4460-4467, 1992
15	MGN026	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1</i> $\Delta(lacZYA-argF)U169 \lambda pir deoR (\phi 80 \Delta lacZM15)$	S. Tinge
	MGN617	SM10 $\lambda pir[thi-1 thr-1 leuB6 supE44 tonA21$ <i>lacY1 recA</i> RP4-2-Tc::Mu $\lambda pir]$ $\Delta asdA4$ $\Delta zhf-2::Tn10$	this study
20	MGN803	MGN617(pMEG226)	this study
	MGN1142	MGN617(pMEG315)	this study
25	<i>S. typhimurium</i>		
	$\chi$ 3761	UK-1, wild-type	R. Curtiss III
30	MGN394	$\Delta cya-27 \Delta crp-28$	P. Sundaram
	MGN431	$\Delta cya-27 \Delta crp-28$ , Mot <sup>+</sup>	P. Sundaram
	MGN510	$\Delta phoP22$ pMEG113 integrant	P. Sundaram
35	MGN806	$\chi$ 3761 pMEG226 integrant	this study
	MGN807	$\Delta cya-28::rfb078$	this study
	MGN868	$\Delta cya-28::rfb078 \Delta crp-28$	this study
40	MGN996	$\Delta cya-28::rfb078 \Delta crp-28$ , Mot <sup>+</sup>	this study
	MGN1175	MGN431 $\Delta asd::xylE729$ (pYA292)	this study

	Strain	Relevant genotype	Source or reference
5	<b><i>S. typhimurium</i></b>		
	MGN1180	$\Delta cya-28::rfb078 \Delta crp-28 \Delta rfc$ , Mot <sup>+</sup>	this study
10	MGN1181	$\Delta cya-28::rfb078 \Delta crp-28 \Delta rfc$ , Mot <sup>+</sup>	this study
	MGN1182	$\Delta cya-28::rfb078 \Delta crp-28 \Delta rfc$ , Mot <sup>+</sup>	this study
15	MGN1183	$\Delta cya-28::rfb078 \Delta crp-28 \Delta rfc$ , Mot <sup>+</sup>	this study
	MGN1184	$\Delta cya-27 \Delta crp-28 \Delta rfc$ , Mot <sup>+</sup>	this study
	MGN1185	$\Delta cya-27 \Delta crp-28 \Delta rfc$ , Mot <sup>+</sup>	this study
20	MGN1717	MGN1180 $\Delta asd::xylE729$	this study
	MGN1718	MGN1717 (pYA292)	this study
25	MGN1720	MGN1717 (pMEG287)	this study
	<b>Plasmids</b>		
	pYA292	Expression vector with p15a ori, Ptrc promoter, Asd <sup>+</sup>	R. Curtiss
30	pYA3255	pYA3174 (Cosmid cloning vector with pSC101 ori Ap <sup>r</sup> ) carrying <i>rfb</i> from $\chi 7122$	Brown et al., Proc. Nat. Acad. Sci. USA Vol. 93 pp. 11149-11154, 1996
35	pMEG055	pYA292 carrying <i>E. coli</i> -derived <i>iutA</i>	this study
	pMEG113	<i>pir</i> -dependent suicide vector carrying $\Delta crp-28$ , Tet <sup>r</sup>	P. Sundaram
40	pMEG219	<i>pir</i> -dependent "suicide" cosmid vector carrying $\Delta cya-27$ , Tet <sup>r</sup> , Kan <sup>r</sup>	this study
	pMEG226	pMEG219 carrying <i>rfb</i> from $\chi 7122$ ( $\Delta cya-28::rfb078$ )	this study
45	pMEG387	pYA292 carrying <i>E. coli</i> -derived type I fimbriae	this study

pMEG315 pMEG149 (*pir*-dependent suicide vector, *sacBR*, this study  
Ap<sup>r</sup>, provided by S. Tinge) carrying *Δrfc*  
The 078 *rfb/rfc* gene cluster was obtained from cosmid clone pYA3255,  
which contains the *rfb* region of APEC strain  $\chi$ 7122 (Brown et al., *Proc. Natl. Acad.*  
5 *Sci. U.S.A.* 93:11149-11154, 1996) inserted into the *Bam*HI cloning site of the cosmid  
vector pYA3174. Restriction analysis of pYA3255 revealed the presence of a *Not*I  
site within the cloned  $\chi$ 7122 DNA in addition to two *Not*I sites that flank the *Bam*HI  
cloning site (Brown et al., *supra*). Therefore, cosmid pYA3255 was partially digested  
with *Not*I and ligated to *Not*I/*Pvu*II-digested pMEG219 to yield cosmid pMEG226,  
10 which has the *rfb* region from APEC strain  $\chi$ 7122 inserted into the  $\Delta$ *cya*-27 allele of  
cosmid pMEG219. Cosmid pMEG226 was then introduced into the *E. coli* strain  
MGN617 by electroporation to produce MGN803 (Table 1). Strain MGN617 has a  
deletion in the *asd* gene, which results in a requirement for diaminopimelic acid  
(DAP) (Schleifer et al., *Bacteriol Rev.* 36:407-477, 1972) and it can support the  
15 replication of pMEG226 because it also carries a copy of the *pir* gene (Table 1).

To introduce cosmid pMEG226 into *S. typhimurium*, the *E. coli* donor strain  
MGN803 was mated with wild-type UK-1 *S. typhimurium* strain  $\chi$ 3761 by spotting 50  
 $\mu$ l of an aerated overnight culture of each strain onto an LB agar plate (Miller, H., in  
*Methods in Enzymology*, Vol. 152, p. 147, Berger, S.L. and Kimmel, A.R., eds.,  
20 Academic Press, Inc. 1987) containing 200  $\mu$ g/ml DAP and incubating overnight at  
37°C. The mating mixture was scraped off of the plate with a sterile wire loop and  
suspended in 1 ml of BSG. Cells were spread onto LB plates containing 10  $\mu$ g/ml  
tetracycline and incubated overnight at 37°C. Counter selection against the donor  
strain, MGN803, was accomplished by plating on selective media that did not contain  
25 DAP. Since cosmid pMEG226 cannot replicate in *S. typhimurium*, the only way  
recombinant cells can acquire resistance to tetracycline is if the plasmid integrates  
into the chromosome by homologous recombination. Tetracycline resistant  
transconjugants were screened for production of 078 LPS by slide agglutination using  
anti-078-specific antisera, which was obtained from the *E. coli* Reference Center at  
30 The Pennsylvania State University. One tetracycline resistant, O78<sup>+</sup> isolate,  
designated MGN806 (Table 1), was grown statically overnight at 37°C in LB broth  
and plated onto fusaric acid media (Bochner et al., *J. Bacteriol.* 243:926-933, 1980) to  
counterselect against the integrated plasmid. Fusaric acid resistant colonies were



screened for loss of tetracycline resistance and white color on MacConkey Maltose media. Cya mutants are white on this media because they are unable to ferment maltose (Botsford et al. *supra*), whereas wild-type *S. typhimurium*, which can ferment maltose, produces red colonies. A tetracycline sensitive, Cya<sup>-</sup>, O78<sup>+</sup> isolate was  
5 selected and designated MGN807 (Table 1).

To add a deletion in the *Salmonella crp* gene, strain MGN807 was transduced with P22 phage grown on strain MGN510 (Table 1) as described previously (Maloy, S. R., *Experimental techniques in bacterial genetics*, Jones and Bartlett, Boston, 1990). MGN510 has integrated into its chromosome the plasmid pMEG113 (Table  
10 1), which is a *pir*-dependent, tetracycline resistant suicide vector containing the *S. typhimurium crp* gene with a defined deletion mutation. Transductants were selected on LB plates containing 10 µg/ml tetracycline. One transductant was selected and plated onto fusaric acid medium. Fusaric acid resistant isolates were screened for the Crp<sup>-</sup> phenotype by streaking onto MacConkey Maltose plates containing 2 mM  
15 cAMP. On this media, Cya<sup>-</sup>, Crp<sup>+</sup> strains are red, while Cya<sup>-</sup>, Crp<sup>-</sup> strains are white. One  $\Delta cya::rfbO78 \Delta crp$  isolate was designated MGN868.

Since  $\Delta cya \Delta crp$  strains are non-motile (Yokota et al., *J. Bacteriol.* 103:513-516, 1970), and because anti-flagella antibodies are known to protect against *S. typhimurium* (Yokoyama et al., *Vaccine* 16:388-393, 1998), MGN868 was passaged  
20 through a motility tube (Holt et al., Enrichment and Isolation, in *Methods for General and Molecular Bacteriology*, Gerhardt et al., ed., p. 222, Am. Soc. for Microbiol., Washington, D.C.) filled with motility agar (Difco) to yield a motile variant of MGN 868, which was designated MGN996 (Table 1).

The LPS expressed by MGN807, MGN 868, and MGN996 was examined by  
25 SDS-PAGE and western blot as follows. Cells of each of these strains, as well as of  $\chi 7122$  and MGN431 (Table 1) as controls for O78 LPS and *S. typhimurium* group B LPS, respectively, were grown overnight in LB broth. Cells were adjusted to an equivalent OD<sub>600</sub> and 1 ml of each culture was pelleted by centrifugation. LPS was prepared from the pelleted cells by the method of Hitchcock and Brown (*J. Bacteriol.*  
30 154:269-277, 1983). Samples were run on duplicate 12.5% SDS polyacrylamide gels, transferred to a nitrocellulose membrane, and probed with anti-Group B-specific

antisera (Difco) or anti-078-specific antisera as described (*ECL Western Blotting Protocols*, p. 16-17, Amersham Life Science, 1997). The results are shown in Fig. 2.

As expected, LPS isolated from the *E. coli* strain  $\chi$ 7122 did not react with anti-Group B antibody (Fig. 2A) but did react with the anti-078 antibody (Fig. 2B).

5 Similarly, the *S. typhimurium* control strain MGN431 (*cya*-27 *crp*-28, *Mot*<sup>+</sup>) made LPS that reacted with anti-Group B antibody (Fig. 2A) but did not react with the anti-078 antibody (Fig. 2B). However, LPS isolated from the recombinant *S. typhimurium* strains MGN807, MGN868 and MGN996 reacted with both antibodies (Figs. 2A, 2B) to produce distinctive ladder patterns of high molecular weight bands, indicating these  
10 strains express both *S. typhimurium* group B and *E. coli* 078 O-antigen attached to the *Salmonella* core. The expression of high molecular weight O78 LPS by the recombinant *Salmonella* strains also indicates that the cloned  $\chi$ 7122 *rfb* region in plasmid pYA3255 also contains the *rfc* gene of  $\chi$ 7122.

The average chain length for the O78 LPS expressed in the recombinant *S.*  
15 *typhimurium* strains was approximately the same size as the average chain length of the group B LPS expressed in the same strain (compare MGN807, MGN868 and MGN996 lanes in Figs. 3A and 3B), while O78 expression in  $\chi$ 7122 resulted in a shorter chain length (Fig. 3B). There is a gene whose product is known to regulate LPS chain length which has been assigned several different names by different  
20 groups, *cld* (Bastin et al., *Mol. Microbiol.* 7:724-734, 1993; Bastin et al., *Mol. Microbiol.* 5:2223-2231, 1991), *rol* (Batchelor et al., *J. Bacteriol.* 174:5528-5236, 1992; Batchelor et al., *J. Bacteriol.* 173:5699-5704, 1991), and, more recently, *wzz* (Reeves et al., *Trends Microbiol.* 4:495-503, 1996). It is likely that in recombinant *S. typhimurium* strains MGN807, MGN 868, and MGN996, the O78 LPS chain length is  
25 determined by the *S. typhimurium wzz* gene.

### Example 2

This example illustrates the ability of the recombinant *S. typhimurium* strain MGN996 ( $\Delta$ *cya*-28::*rfb*O78,  $\Delta$ *crp*-28, *Mot*<sup>+</sup>) to colonize chickens and protect them  
30 from challenge with the wild-type APEC strain  $\chi$ 7122.

All chickens used in the experiments described in Examples 2-4 were White Leghorns hatched from fertile eggs from specific-pathogen-free (SPF) chickens

obtained from Specific Pathogen Free Avian Services (SPAFAS, Roanoke, IL). The eggs were incubated and hatched in a Humidaire incubator/hatcher located in the Department of Biology at Washington University in St. Louis, MO.

In this experiment birds were vaccinated twice: once at day-of-hatch and again  
5 at 14 days of age. Chicks were inoculated at day-of-hatch with  $4.6 \times 10^6$  CFU of MGN996 per chick by coarse spray. Coarse spray vaccination was performed by placing 10-15 chicks into a Plexiglas spray box and delivering the inoculum using a stainless steel Sure Shot Model A sprayer (Milwaukee Sprayer Manufacturing Co., Inc., Milwaukee, WI) pressurized to 100 psi. Practice trials were performed in  
10 advance to determine the appropriate conditions to deliver 0.3 ml/chick. Twenty six chicks were vaccinated with MGN966 and twelve chicks were mock vaccinated with BSG. After inoculation, the chicks were transferred to Horsfall isolators and provided food and water *ad libitum* after day of hatch vaccinations.

At day 10, three chicks, chosen at random from the vaccinated group, were  
15 euthanized by CO<sub>2</sub> inhalation and then immediately necropsied to assess colonization. Samples of lung, liver, spleen, bursa of Fabricius and cecal contents were taken from each bird, placed into individual stomacher bags, and 5 ml of BSG was added to each sample. Tissues were homogenized in a Seward Stomacher 80 Laboratory Blender. Then, 0.1 ml of homogenized sample was plated onto MacConkey agar base (Difco)  
20 supplemented with 1% maltose, for spleen, liver and lung, or Brilliant Green agar (Difco) containing 35 µg/ml novobiocin for bursa of Fabricius samples and cecal contents. Plates were incubated overnight at 37°C. At least one typical colony from each plate was further analyzed to confirm expression of the appropriate LPS type(s) by slide agglutination. For cases in which no colonies were obtained from direct  
25 plating, 0.5 ml of homogenized tissue sample was added to 4.5 ml Hajna broth and incubated overnight at 42°C as a secondary enrichment. A loopful of the Hajna-enriched culture was streaked onto Brilliant Green Agar and incubated overnight at 37°C. The MGN996 strain was found in samples of all tissues examined.

On day 14, vaccinated birds were boosted with  $3.8 \times 10^7$  CFU of MGN996 in  
30 a volume of 0.2 ml by oral gavage using a feeding cannula attached to a 1 cc syringe. On day 28, all birds were challenged with  $7.5 \times 10^7$  CFU of *E. coli* strain  $\chi$ 7122 in a

volume of 0.5 ml by the intratracheal route and four days later were euthanized by CO<sub>2</sub> inhalation and then necropsied.

The necropsied birds were scored for lesions associated with APEC infection. In this experiment and those described in Examples 3-4, analysis of the lesion scores was done using the Wilcoxon-Mann-Whitney U test (Zar, J.H., *Biostatistical Analysis*, Prentice-Hall, Inc., Englewood Cliffs). In this experiment, a  $\chi^2$  analysis was used to determine differences between groups based on the number of birds showing no lesions (lesion score of 1) and the results are shown below in Table 2.

**Table 2. Mean lesion scores of chickens vaccinated with MGN996 vs non-vaccinates after challenge with APEC strain  $\chi$ 7122<sup>1</sup>.**

Group	# of Birds with Each Lesion Score					Mean Score <sup>2</sup>
	1	2	3	4	5	
BSG	1	0	1	8	2	3.8 <sup>a</sup>
MGN996	10	2	1	7	1	2.4 <sup>b</sup>

<sup>1</sup> Birds were scored as follows: 1. live birds, no lesions 2. Live bird with cloudy air sacs 3. Live bird with only one type of lesion (e.g. perihepatitis only) 4. Live bird with multiple lesions 5. Dead or moribund bird with lesions typical of colibacillosis (perihepatitis, pericarditis, air sacculitis, severe dehydration).

<sup>2</sup> a different from b (p=0.0157)

The mean lesion scores indicate that birds vaccinated with MGN996 were significantly protected from challenge when compared to non-vaccinated control birds ( $\chi^2=5.3$ , p=0.02). In addition, the vaccinated group showed a significant reduction in overall mean lesion scores (p=0.0157). These results show that delivery of *E. coli* LPS by an attenuated *S. typhimurium* also expressing group B O-antigen provides chickens with significant protection from challenge with wild-type *E. coli*.

### Example 3

This example illustrates the effect of removing expression of the *Salmonella* O-antigen on the ability to colonize various chick tissues.

To eliminate expression of group B O-antigen by the recombinant *S. typhimurium* vaccine strain, a defined deletion was made in the *Salmonella rfc* gene of MGN996 ( $\Delta cya::O78 \Delta crp$ ; Table 1) and MGN431 (a defined  $\Delta cya \Delta crp$  deletion control strain, Table 1). Strain MGN996 or MGN 431 was mated with strain  
5 MGN1142 (Table 1) essentially as described in Example 1 with selection for ampicillin resistance. MGN1142 contains a *pir*-dependent suicide vector carrying an *S. typhimurium* mutant *rfc* gene. A transconjugant from each mating was grown overnight in LB broth containing 50  $\mu$ g/ml ampicillin and plated onto LB agar plates minus NaCl supplemented with 5% sucrose and incubated for 48 hours at room  
10 temperature. Isolates were screened for the Rfc phenotype by cross streaking against P22 phage. As discussed above, in this assay mutants deficient in *S. typhimurium* O-antigen polymerase are resistant to P22, while isolates expressing functional *S. typhimurium* O-antigen polymerase are sensitive.

Several independent  $\Delta rfc_{St}$  (denotes the *rfc* gene of *S. typhimurium*) isolates  
15 were obtained and designated MGN1180, MGN1181, MGN1182, and MGN1183, which are derived from MGN996; and strains MGN1184 and MGN1185, which are derived from the  $\Delta cya \Delta crp$  *S. typhimurium* strain MGN431. See Table 1. The LPS made by these strains was isolated and analyzed for group B and O78 O-antigen by western blot as described above in Example 1 and the results are shown in Figure 3.

20 As expected, LPS made by the  $\Delta cya \Delta crp$  control strain MGN431 and its  $\Delta rfc_{St}$  derivatives MGN 1184 and MGN1185 reacted with anti-group B LPS antibody (Fig. 4A) and did not react with anti-O78 LPS antibody (Fig. 4B). However, the LPS isolated from the  $\Delta rfc_{St}$  strains produced only low molecular weight species when probed with anti-group B LPS antibody (Fig. 4A) indicating they did not express full-  
25 length group B LPS. When probed with anti-O78 LPS antibody, LPS made by MGN996 and its  $\Delta rfc_{St}$  derivative strains (MGN1180, MGN1181, MGN1182, and MGN1183) produced a pattern indicative of complete O78 *E. coli* O-antigen (Fig. 4B). The O78-specific LPS pattern in strain MGN1180 (Fig. 4B) was similar to the group B LPS chain length in MGN996 (Fig. 4A), while the O78-specific LPS pattern  
30 in MGN1181 (Fig. 4B) was more similar to the O78 LPS pattern seen in the wild-type O78 *E. coli* strain,  $\chi$ 7122 (Fig. 4B). The difference in LPS patterns was unexpected, since the two strains have apparently identical genotypes (Table 1). Strains

MGN1180, MGN1181 and MGN1184 were chosen for further study as possible vaccines against *E. coli*  $\chi$ 7122.

Ten chicks per strain were inoculated at day-of-hatch by oral gavage with MGN996 ( $6.9 \times 10^7$  CFU/chick), MGN1180 ( $4.0 \times 10^7$  CFU/chick), MGN1181 ( $2.4 \times 10^7$  CFU/chick), MGN1184 ( $1.3 \times 10^7$  CFU/chick), or MGN431 ( $7.8 \times 10^7$  CFU/chick). The vaccinated birds were placed into isolators and necropsied at day 10 as described above. Samples of lung, liver, spleen, bursa of Fabricius and cecal contents were collected, processed and assessed for colonization as described in Example 2 and the results are shown in Table 3 below.

**Table 3. Colonization of chickens by recombinant  $\Delta cya \Delta crp \Delta rfc$  *S. typhimurium* vaccine candidates and control strains (% birds positive)**

Strain	Genotype	Lung	Spleen	Liver	Bursa	Ceca
MGN996	<i>cya::rfb</i> 078 $\Delta crp$	100%	100%	100%	100%	100%
MGN1180	<i>cya::rfb</i> 078 $\Delta crp \Delta rfc$	80%	100%	100%	100%	100%
MGN1181	<i>cya::rfb</i> 078 $\Delta crp \Delta rfc$	70%	100%	100%	100%	100%
MGN1184	$\Delta cya \Delta crp \Delta rfc$	20%	50%	70%	100%	100%
MGN431	$\Delta cya \Delta crp$	77%	100%	66%	100%	100%

The results indicate that all of the  $\Delta rfc_{SI}$  strains tested, except MGN1184, are capable of colonizing a large percentage of the birds' lung, spleen, liver, bursa of Fabricius and ceca. Like control strains MGN431 and MGN996, strains MGN1180 and MGN1181 were found to colonize 100% of the spleens of vaccinated birds. Strain MGN1184 has a semi rough phenotype due to the  $\Delta rfc$  mutation. It has been reported that LPS-deficient *S. typhimurium* strains exhibit reduced colonization of chickens (Craven et al., *Avian Dis.* 38:401-408, 1994). Bird to bird variation notwithstanding, the results obtained in this experiment were consistent with the fact that colonization by MGN1184 was severely impaired, confirming a role of complete LPS O-antigen in colonization. However, expression of O78 *E. coli* O-antigen in an Rfc background (MGN1180 and MGN1181) resulted in a significant increase in the

percentage of birds colonized when compared to MGN1184, suggesting that it would be useful to evaluate these strains for their ability to protect against *E. coli* challenge.

#### Example 4

5 This example illustrates the efficacy of a recombinant *S. typhimurium* strain which expresses O78 *E. coli* O-antigen but does not express group B O-antigen in protecting chicks against disease caused by infection with the O78 APEC strain,  $\chi$ 7122.

10 Thirty-five chicks per strain were inoculated at day-of-hatch by oral gavage with either  $6.5 \times 10^7$  CFU of MGN996 ( $\Delta cya-28::rfbO78 \Delta crp-28$ , Mot<sup>+</sup>) or  $7.7 \times 10^7$  CFU of MGN1180 ( $\Delta cya-28::rfbO78 \Delta crp-28 \Delta rfc$ , Mot<sup>+</sup>). Twenty chicks were mock vaccinated with BSG. At day 10, five chicks from each inoculated group were necropsied to assess colonization as described in Example 2. The colonization results were similar to those seen in Experiment 2 (data not shown). On day 14, birds  
15 previously inoculated with MGN996 were boosted with  $7.4 \times 10^7$  CFU of MGN996 and birds previously vaccinated with MGN1180 were boosted with  $6.4 \times 10^7$  CFU of MGN1180. All birds were leg banded on day 24. To assess post-vaccination serum antibody responses, serum was collected by wing vein puncture from half of the birds on day 27 and the other half on day 28. Half the birds in each group were challenged  
20 on day 30 and the remaining birds were challenged on day 31. The challenge dose was  $9.6 \times 10^7$  CFU of *E. coli* strain  $\chi$ 7122. Two control birds died prior to bleeding, so only 18 control birds were bled and challenged. Necropsies were performed on all birds four days post-challenge to assess colonization by  $\chi$ 7122 as described in Example 2, except that heart blood was also collected. The challenge strain was  
25 isolated from all birds, but was primarily found only in the ceca of birds vaccinated with MGN996 or MGN1180. Lesion scoring data from the two necropsy days were pooled for analysis and the results are shown in Table 4 below.

**Table 4. Mean lesion scores of chickens vaccinated with *S. typhimurium* strains MGN996, MGN1180 or non-vaccinates after challenge with APEC strain  $\chi$ 7122<sup>1</sup>.**

Treatment	# of Birds with Each Lesion Score					Mean Lesion Score <sup>1</sup>
	1	2	3	4	5	
None	0	4	6	8	0	3.2 <sup>a</sup>
MGN996	9	8	8	5	0	2.3 <sup>b</sup>
MGN1180	14	10	4	2	0	1.8 <sup>c</sup>

Lesions scores were determined as described in Table 2.

<sup>1</sup> a different from b,  $p = 0.0064$

a different from c,  $p < 0.0001$

- 15 The results confirm the efficacy of strain MGN996 and indicate that MGN1180 may be a superior *E. coli* vaccine, since the mean lesion score for birds vaccinated with MGN1180 are lower than the mean lesion score for MGN996 vaccinates, although the difference was not significant ( $p=0.08$ ).
- 20 Serum from each bird was evaluated for antibody responses directed against *E. coli* O78 LPS and *S. typhimurium* LPS by enzyme-linked immunosorbent assay (ELISA). Wells of an Immulon-I flat bottom plate were coated with 100  $\mu$ l of either *E. coli* LPS (Sigma) or *S. typhimurium* LPS (Sigma) prepared at 2.5  $\mu$ g/ml in 0.2% TCA (trichloroacetic acid; Sigma) for 2 hours at 37°C (Hardy, 1994). Following coating, the plate was washed three times with a TBS (Tris Buffered Saline)/0.1% tween 20 solution. Diluted sera (1:100) were added to duplicate wells and the plate
- 25 incubated at 37°C for 1 hour. This step was followed by three washes with TBS/0.1% tween 20 solution. Bound IgG was detected by the addition of a 1:30,000 diluted goat anti-chicken IgG HRP conjugated antibody (KPL). Plates were again washed with TBS/0.1% tween 20 to remove unbound detection antibody. Washing was followed
- 30 by development with OPD (o-phenylenediamine dihydrochloride) tablets (Sigma) in phosphate-citrate buffer with sodium perborate. After 15 minutes at 37°C, the reaction was stopped by the addition of 3N HCl to each well. Plates were read at 490nm using a BT2000 Microkinetics Reader (FisherBiotech). To detect bound IgM and IgA, the plate was first blocked using 1:5 diluted normal rabbit sera for 45
- 35 minutes at 37°C followed by three washes of TBS/0.1% tween 20. After blocking,



1:5000 diluted goat anti-chicken IgM or IgA (Immunovision) was added to each well and the plate incubated at 37°C for an additional 45 minutes. After three washes in TBS/0.1% tween 20 to remove the unbound antibody, 1:5000 diluted rabbit anti-goat IgG alkaline phosphate conjugated antibody (Sigma) was added to each well. The plate was again incubated at 37°C for 45 minutes. After removal of unbound conjugated antibody, a detection step of p-NPP (P-nitrophenylphosphate in diethanolamine; Sigma) substrate solution was added to each well and the plate incubated for 30 minutes at 37°C. The reaction was then stopped by the addition of 3M NaOH and the plate read at 405nm. Birds were considered seropositive if an OD value greater than 0.2 was observed. Controls included prebleeds from 12 birds and wells containing conjugated antibody only. The results are shown in Tables 5 and 6 below.

**Table 5. Percent Seropositive for 078 LPS, Pre- and Post-challenge**

Isotype	TREATMENT GROUPS					
	Controls		MGN996 (Rfc <sup>+</sup> )		MGN1180 (Rfc)	
	Pre	Post	Pre	Post	Pre	Post
IgM	0	88	53	100	80	97
IgG	0	17	20	77	57	83
IgA	0	17	30	87	70	87

**Table 6. Percent Seropositive for group B LPS, Pre- and Post-challenge**

Isotype	TREATMENT GROUPS					
	Controls		MGN996 (Rfc <sup>+</sup> )		MGN1180 (Rfc)	
	Pre	Post	Pre	Post	Pre	Post
IgM	0	17	93	100	30	57
IgG	0	6	27	67	7	17
IgA	0	0	70	87	10	20

Approximately 50% of the birds vaccinated with MGN996 were seropositive for O78-specific IgM antibodies, while only 20-30% were positive for O78-specific IgG and IgA antibodies pre-challenge. By contrast, 80% of the birds vaccinated with the Rfc<sub>St</sub><sup>-</sup> strain, MGN1180, were positive for O78-specific IgM antibodies pre-challenge, with 57% and 70% of the birds seropositive for IgG and IgA O78-specific antibodies, respectively. These results indicate a further maturation of humoral immune responses generated by vaccination with MGN1180 than with the Rfc<sub>St</sub><sup>+</sup> strain MGN996. Although post-challenge seroconversion was similar between the groups, the OD<sub>405</sub> values of 85% (22/26) of the IgA seropositive birds vaccinated with MGN1180 were greater than 0.5 as compared to only 42% (11/26) of the birds vaccinated with MGN996. The high percentage of mock-vaccinated birds seropositive for anti-O78 IgM may be due to restimulation of memory B cells reactive to a common *E. coli* antigen present in the O78 LPS preparation (e.g. LPS core). As expected, MGN996 vaccinates gave a stronger humoral antibody response to *S. typhimurium* LPS than did MGN1180 vaccinates, since MGN1180 produces *S. typhimurium* LPS having only a single sugar unit of the O-antigen, which may be masked by the O78 LPS. This speculation is based upon the observation that strain MGN1180 does not agglutinate in the presence of group B specific antisera, while strain MGN1184 ( $\Delta cya \Delta crp \Delta rfc$ ) does agglutinate, albeit more weakly than a *S. typhimurium* strain expressing complete LPS O-antigen.

Overall there was a strong correlation between a robust pre-challenge antibody response and a low lesion score, although there were a few birds that did not mount a detectable pre-challenge antibody response that were protected, and there were a few birds that mounted a detectable pre-challenge antibody response but were scored as not being protected. Most of the birds vaccinated with MGN1180 were seropositive for O78 antibodies (24/30) before challenge, while fewer birds (17/30) from the MGN996 group were seropositive.

#### Example 5

This example illustrates the vaccination of chicks with a recombinant vaccine *S. typhimurium* strain also containing an Asd<sup>+</sup> vector that can be used to express additional heterologous gene products.

The Asd<sup>+</sup> vector pYA292 (Table 1) was introduced into MGN1717 (Table 1) by electroporation to produce strain MGN1718 [(*Δcya-28::rfbO78 Δcrp-28 Δrfc1 Δasd::xylE729*)/ pYA292, Mot<sup>+</sup>]. The chickens used in this Example were all hatched from fertile eggs from specific-pathogen-free (SPF) chickens obtained from Sunrise Farms (Catskill, NY). Twenty chicks were mock-vaccinated with BSG and 29 chicks were vaccinated at day-of-hatch with  $5.6 \times 10^7$  CFU of MGN1718 by oral gavage as described above. On day 14, vaccinated birds were boosted with  $5.2 \times 10^7$  CFU of MGN1718 by oral gavage and on day 28 all birds were challenged with  $5 \times 10^7$  CFU of  $\chi$ 7122 as described above. Four days later the birds were euthanized, necropsied, and scored for lesions as described above except a new, more sensitive scoring system was used, which is listed in Table 7 below and the mean lesion scores using this scoring system are shown below in Table 8.

Table 7

Method for scoring lesions:

5	<b>Air sacs (thoracic)</b>	
	Normal	0
	Mild cloudiness and thickness	1
	Moderate cloudiness, and thickness accompanied by serous exudates and fibrin spots	2
10	Extensive cloudiness and thickness accompanied by muco- or fibrino-purulent exudate	3
	<b>Heart and pericardium</b>	
	Normal	0
15	Turbidity and presence of excessive fluid in the pericardial cavity	1
	Acute pericarditis	2
	<b>Liver</b>	
20	Normal	0
	Decolorized and/or slight amounts of fibrinous exudates	1
	Marked perihepatitis	2
	<u>Bacteriological examinations:</u>	
25	<b>Re-isolation of <i>E. coli</i></b>	
	after incubating air sac-swabbed sample in BHI	1
	from direct streak for air sacs	2
	after incubating pericardial fluid- or heart-swabbed sample in BHI	1
30	from direct streak from pericardial or heart sample	2
	from incubating liver-swabbed sample in BHI	1
	from direct liver streak	2
35	Maximum possible points per bird = 13.	

**Table 8 Mean Lesion Scores for  $\chi$ 7122 challenge.**

Treatment	# of Birds with each Lesion Score										Mean Lesion Score <sup>1</sup>
	0	1	2	3	4	5	6	7	8	9	
Non-vac	1	2	1	2	2	2	2	2	2	4	5.30 <sup>a</sup>
MGN1718	8	8	9	1	1	0	0	2	0	0	1.62 <sup>b</sup>

<sup>1</sup> a different from b (p< 0.0001)

5 To assess whether the *S. typhimurium* carrier strain is contributing to the protection against challenge with the APEC strain, a very similar experiment was done using MGN1175 (Table 1), which is genetically very similar to MGN1718 but lacks the O78 *rfb* gene cluster. The mean lesion scores for mock-vaccinated controls or vaccinated birds following challenge with  $7.5 \times 10^7$  CFU of  $\chi$ 7122 are shown in

10 Table 9 below.

**Table 9 Mean Lesion Scores for  $\chi$ 7122 challenge**

Treatment	# of Birds with each Lesion Score										Mean Lesion Score
	0	1	2	3	4	5	6	7	10	11	
Non-vac	3	1	2	2	2	6	2	1	0	1	3.95
MGN1175	3	3	4	8	2	6	1	1	2	0	3.53

15 The mean lesion score for vaccinated birds was not significantly different than that for the mock-vaccinated control birds. This result indicates that the protection against the APEC  $\chi$ 7122 strain provided by the recombinant *S. typhimurium* vaccine strains MGN1718 and MGN1180 strains was due to the expression of O78 *E. coli* O-antigen.

20

**Example 6**

This example illustrates the construction and efficacy of one embodiment of the O-78 vaccine strain which also expresses a fimbrial operon of an O1:K1:H7 APEC strain.

25 Fimbrial operons are cell surface structures which have been associated with virulence in APEC strains (Dozios et al., *Infect. Immun.* 60:2648-2656, 1992; Dozios,

- et al., *Avian Dis.* 38:231-239, 1994; Wooley et al., *Av. Dis.* 36:679-684, 1992), and type I fimbriae, in particular, have been implicated important for colonization of avian tracheal epithelial cells by avian pathogenic *E. coli* (APEC) strains (Gyimah et al., *Avian Dis.* 32:74-78, 1988). The genes involved in type 1 fimbriae production have been cloned from an O1:K1:H7 APEC isolate, inserted into an Asd<sup>+</sup> plasmid vector (pYA292) and expressed constitutively. A genetic map of the resulting plasmid, pMEG287, is shown in Figure 4. Plasmid pMEG287 was introduced into the recombinant *S. typhimurium* strain MGN1717 (Table 1), to yield MGN1720 [ $\Delta$ cya-28:rfb O78  $\Delta$ crp-28  $\Delta$ rfa1  $\Delta$ asd::xylE729]/pYA292, Mot<sup>+</sup>. Expression of type I fimbriae in strain MGN1720 was confirmed by a yeast agglutination assay (Korhonen, T. K., *FEMS Microbiol. Lett.* 6:421-425, 1979), using aerated overnight cultures grown at 37°C. Agglutination was mannose-sensitive, as expected for type I fimbriae, and the control strain, MGN1718, did not agglutinate yeast cells. Although wild-type *S. typhimurium* strains express type I fimbriae, the result with strain MGN1718 is consistent with the fact that expression of type I fimbriae expression is regulated by  $\Delta$ cya  $\Delta$ crp, and are therefore only poorly expressed in Cya<sup>-</sup> Crp<sup>-</sup> strains (Saier et al., *J. Bacteriol.* 134:356-358, 1978), and only after two days of static growth at 37°C.

- The efficacy of strains MGN1718 and MGN1720 were evaluated in chickens essentially as described above using a challenge dose of  $5 \times 10^7$  CFU and the results are shown below in Table 10.

**Table 10. Mean Lesion Scores for  $\chi$ 7122 challenge.**

Treatment	# of Birds with each Lesion Score										Mean Lesion Score <sup>1</sup>
	0	1	2	3	4	5	6	7	8	9	
Non-vac	1	2	1	2	2	2	2	2	2	4	5.30 <sup>a</sup>
MGN1718	8	8	9	1	1	0	0	2	0	0	1.62 <sup>b</sup>
MGN1720	6	16	6	0	0	1	0	0	0	0	1.14 <sup>b</sup>

p < 0.0001

<sup>1</sup> a different from b (p < 0.0001)

These results show that the addition of type I fimbriae results in lower lesion scores, although not statistically significant. While the addition of type I fimbriae to the

vaccine strain did not enhance protection against O78 challenge, it may provide cross-protection against other APEC serotypes.

Other fimbriae that may be used are P type fimbriae, including F11, which have been associated with APEC strains (van den Bosch, et al., *Infect. Immun.* 61:800-806, 1993).

#### Example 7

This example illustrates the construction of an embodiment of the O78 vaccine strain which also expresses an *E. coli* iron-regulated outer membrane protein.

The ability to scavenge iron is an important survival trait for pathogens because the availability of iron is limiting in the animal host environment (Litwin et al., *Clin. Microbiol. Rev.* 6:509-518, 1993; Peighambari, et al., *Avian Dis.* 39:116-124, 1995). A number of iron-responsive genes encode outer membrane proteins (OMPs), with most of these acting as receptors for iron-chelating compounds (siderophores) produced by the bacteria or the host (Bagg, et al., *Microbiol. Rev.* 51:509-518, 1987).

Iron-regulated OMPs are antigenic and antibodies against *E. coli* iron-regulated OMPs have been detected in healthy humans, rabbits, mice and guinea pigs (Griffiths, et al., *Infect. Immun.* 47:808-813, 1985). Antibodies raised against *E. coli* iron-regulated OMPs passively protect turkeys against subsequent challenge with an avian pathogenic O78 *E. coli* (Bolin, et al., *Infect. Immun.* 55:1239-1242, 1987), indicating that these proteins are protective antigens.

An excellent candidate for a vaccine antigen is the iron-responsive 78-kDa OMP IutA. IutA is the receptor for aerobactin and cloacin (Bagg, et al., *Microbiol. Rev.* 51:509-518, 1987). Synthesis of aerobactin is directly correlated with *E. coli* virulence in humans and mammals (Litwin et al., *Clin. Microbiol. Rev.* 6:509-518, 1993; Payne, S. M., *Crit. Rev. Microbiol.* 16:81-111, 1988.), as well as in poultry (Dho et al., *Avian Dis.* 28:1016-1025, 1984; Emery et al., *Avian Dis.* 36:504-511, 1992; Lafont et al., *Infect. Immun.* 55:193-197, 1987; Linggood et al., *J. Gen. Microbiol.* 133:835-842, 1987; Wooley et al., *Av. Dis.* 36:679-684, 1992). Aerobactin is a component of a high affinity iron uptake system (Bagg, et al., *Microbiol. Rev.* 51:509-518, 1987), which may account for its prevalence among pathogenic organisms. A number of studies of avian *E. coli* isolates have found that greater than

80% of pathogenic isolates produce aerobactin (Dho et al., *supra*; Emery et al., *supra*; Lafont et al., *supra*; Yokoyama et al., *supra*), while it is rare among non-pathogenic strains. The genes for aerobactin synthesis are most often found on ColV plasmids, and therefore are tightly linked to colicin V production (Bagg et al., *supra*).

5        An *IutA* clone from pColV-K30 is available as plasmid pFS8 (Krone et al., *J. Bacteriol.* 153:716-721, 1983). The *iutA* gene was subcloned from pFS8 into an *Asd*<sup>+</sup> plasmid vector pYA292 to create pMEG055 (Fig. 5), and introduced into a  $\Delta$ *asd* derivative of *S. typhimurium* strain MGN996. Results of western blot analysis indicates that the *IutA* protein is constitutively expressed and that expression is stable  
10 over 50 generations in L broth.

Other outer membrane proteins that may be expressed include the iron-regulated outer membrane proteins, FepA, FecA, FhuA, FecA, and a protein involved in serum resistance, Iss.

15        All references cited in this specification either *supra* or *infra* are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by their authors and no admission is made as to the accuracy or pertinency of the references or that any reference is material to patentability.



What is Claimed is:

1. A vaccine for immunization of birds against an avian pathogenic gram-negative (AP<sub>G-N</sub>) microbe comprising live cells of a recombinant *Salmonella* strain expressing an O-antigen of the AP<sub>G-N</sub> microbe, the recombinant *Salmonella* strain having an *rfb/rfc* gene cluster of the AP<sub>G-N</sub> microbe stably integrated into the  
5 *Salmonella* chromosome and having a mutation in the *Salmonella rfb* gene cluster or in the *Salmonella rfc* gene which inactivates expression of *Salmonella* O-antigen, wherein the recombinant *Salmonella* strain is an attenuated mutant of a virulent *Salmonella* strain.
2. The vaccine of claim 1, wherein the AP<sub>G-N</sub> microbe is an avian pathogenic *Escherichia coli* (APEC) strain.
3. The vaccine of claim 2, wherein the integrated APEC *rfb/rfc* gene cluster comprises an attenuating mutation in a *Salmonella* gene selected from the group consisting of *pab*, *pur*, *aro*, *asd*, *dap*, *nadA*, *pncB*, *galE*, *pmi*, *fur*, *rpsL*, *ompR*, *htrA*, *hemA*, *cdt*, *cya*, *crp*, *phoP*, *phoQ*, *rfc*, *poxA*, and *galU*.
4. The vaccine of claim 3, wherein the attenuating mutation is a defined deletion/insertion mutation in the *Salmonella cya* gene.
5. The vaccine of claim 4, wherein the recombinant *Salmonella* strain also has an attenuating mutation in the *Salmonella crp* gene.
6. The vaccine of claim 5, wherein the APEC strain has a serotype of O1, O2, O35 or O78.
7. The vaccine of claim 1, wherein the recombinant *Salmonella* strain also has a recombinant polynucleotide encoding a desired gene product.
8. The vaccine of claim 7, wherein the desired gene product is an antigen from an avian pathogenic organism.
9. The vaccine of claim 8, wherein the avian pathogenic organism is an avian pathogenic *Escherichia coli* (APEC) strain and the antigen is a fimbriae or an iron-regulated outer membrane protein of the APEC strain.
10. A method for immunizing a bird against an avian pathogenic gram-negative (AP<sub>G-N</sub>) microbe, the method comprising administering to the bird an immunologically effective amount of a vaccine comprising live cells of a recombinant *Salmonella* strain expressing an O-antigen of the AP<sub>G-N</sub> microbe, the recombinant

- 5 *Salmonella* strain having an *rfb/rfc* gene cluster of the AP<sub>G-N</sub> microbe stably integrated into the *Salmonella* chromosome and having a mutation in the *Salmonella rfb* gene cluster or in the *Salmonella rfc* gene which inactivates expression of *Salmonella* O-antigen, wherein the recombinant *Salmonella* strain is an attenuated mutant of a virulent strain of a *Salmonella* strain.
11. The method of claim 10, wherein the AP<sub>G-N</sub> microbe is an avian pathogenic *Escherichia coli* (APEC) strain.
12. The method of claim 11, wherein the integrated APEC *rfb/rfc* gene cluster comprises an attenuating mutation in a *Salmonella* gene selected from the group consisting of *pab*, *pur*, *aro*, *asd*, *dap*, *nadA*, *pncB*, *galE*, *pmi*, *fur*, *rpsL*, *ompR*, *htrA*, *hemA*, *cdt*, *cya*, *crp*, *phoP*, *phoQ*, *rfc*, *poxA*, and *galU*.
13. The method of claim 12, wherein the attenuating mutation is a defined deletion/insertion mutation in the *Salmonella cya* gene.
14. The method of claim 13, wherein the recombinant *Salmonella* strain also has an attenuating mutation in the *Salmonella crp* gene.
15. The method of claim 14, wherein the APEC strain has a serotype of O1, O2, O35 and O78.
16. The method of claim 10, wherein the recombinant *Salmonella* strain also has a recombinant polynucleotide encoding a desired gene product.
17. The method of claim 10, wherein the bird is a chicken or a turkey.
18. The method of claim 17, wherein the vaccine is administered by coarse spray at day-of-hatch.
19. The method of claim 18, further comprising orally administering to the bird a booster amount of the vaccine.
20. The method of claim 19, wherein the booster amount is administered on day 13, 14, or 15 after day-of-hatch.
21. A vaccine for immunization of birds against at least two avian pathogenic gram-negative (AP<sub>G-N</sub>) microbes, the vaccine comprising a mixture of live cells of first and second recombinant *Salmonella* strains, the first recombinant *Salmonella* strain having an *rfb/rfc* gene cluster of a first AP<sub>G-N</sub> microbe integrated  
5 into the *Salmonella* chromosome and expressing an O-antigen of the first AP<sub>G-N</sub> microbe and the second recombinant *Salmonella* strain having an *rfb/rfc* gene cluster of a second AP<sub>G-N</sub> microbe integrated into the *Salmonella* chromosome and

expressing an O-antigen of the second AP<sub>G-N</sub> microbe, wherein each of the first and second recombinant *Salmonella* strains has a mutation in the *Salmonella rfb* gene cluster or in the *Salmonella rfc* gene which inactivates expression of *Salmonella* O-antigen, and wherein each of the first and second recombinant *Salmonella* strains is an attenuated mutant of a virulent *Salmonella* strain.

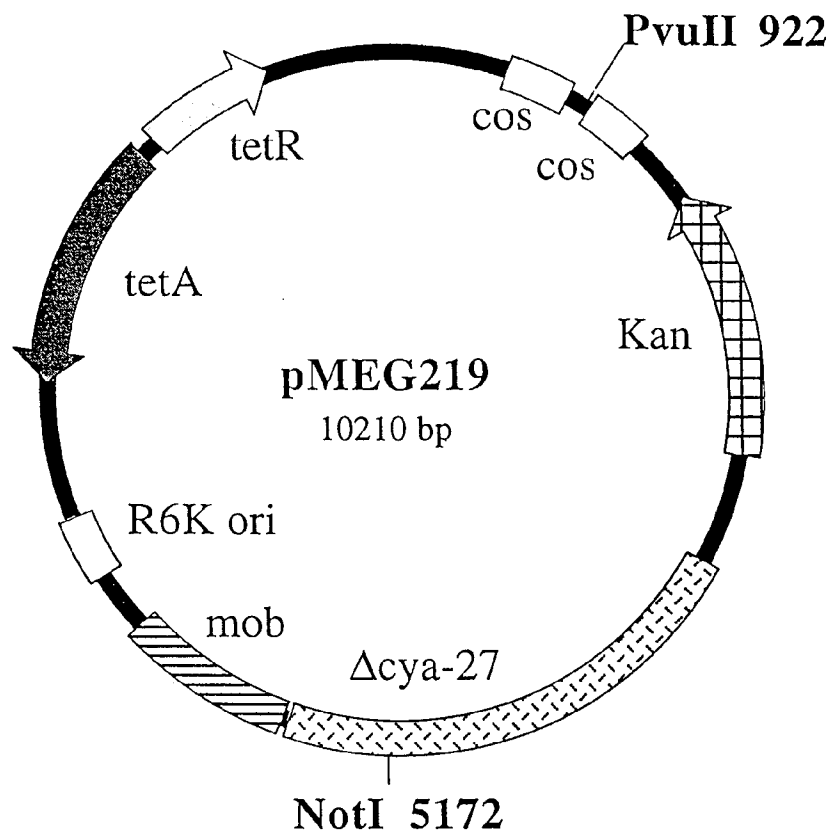
22. A vaccine for immunization of birds against at least two avian pathogenic gram-negative (AP<sub>G-N</sub>) microbes, the vaccine comprising live cells of a recombinant *Salmonella* strain expressing an O-antigen of each of the AP<sub>G-N</sub> microbes, the recombinant *Salmonella* strain having an *rfb/rfc* gene cluster of each of the AP<sub>G-N</sub> microbes stably integrated into the *Salmonella* chromosome and having a mutation in the *Salmonella rfb* gene cluster or in the *Salmonella rfc* gene which inactivates expression of *Salmonella* O-antigen, wherein the recombinant *Salmonella* strain is an attenuated mutant of a virulent *Salmonella* strain.

23. A method of making a vaccine for immunizing a bird against an avian pathogenic gram-negative (AP<sub>G-N</sub>) microbe comprising the steps of:

- (a) selecting a *Salmonella* strain capable of colonizing the bird;
  - (b) integrating into the *Salmonella* chromosome an *rfb/rfc* gene cluster from the AP<sub>G-N</sub> microbe;
  - (c) introducing a mutation into the *Salmonella rfb* gene cluster and/or into the *Salmonella rfc* gene; and
  - (d) isolating recombinant *Salmonella* bacteria which express O-antigen characteristic of the APEC strain but which do not express *Salmonella* O-antigen,
- wherein steps (b) and (c) can be performed in any order.

24. The method of claim 23, wherein the selected *Salmonella* strain is an attenuated mutant of a virulent *Salmonella* strain.

25. The method of claim 24, wherein the selected *Salmonella* strain is a virulent *Salmonella* strain and the method further comprises introducing into the virulent *Salmonella* strain an attenuating mutation in a *Salmonella* gene selected from the group consisting of *pab*, *pur*, *aro*, *asd*, *dap*, *nadA*, *pncB*, *galE*, *pmi*, *fur*, *rpsL*, *ompR*, *htrA*, *hemA*, *cdt*, *cya*, *crp*, *phoP*, *phoQ*, *rfc*, *poxA*, and *galU*, and then isolating mutants having attenuated virulence as compared to the virulent *Salmonella* strain.

**FIGURE 1**

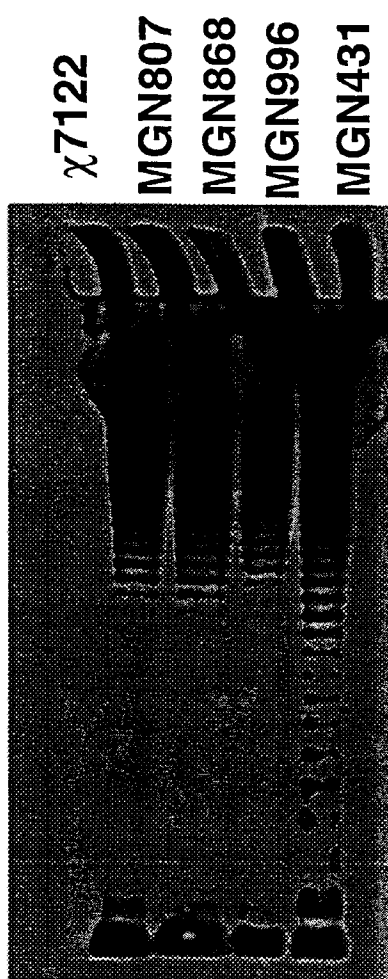


FIGURE 2A

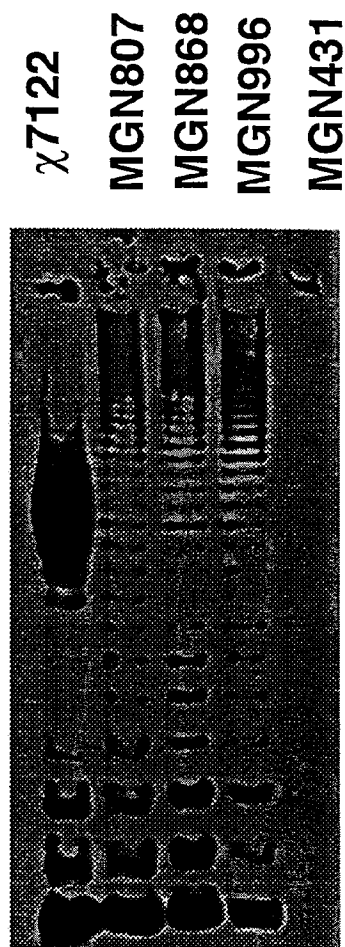


FIGURE 2B

MGN431  
MGN1184  
MGN1185  
χ 7122  
MGN996  
MGN1180  
MGN1181  
MGN1182  
MGN1183

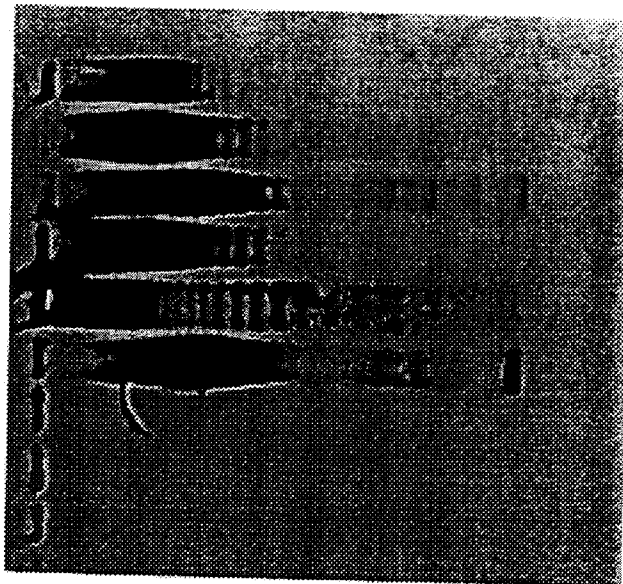


FIGURE 3B

MGN431  
MGN1184  
MGN1185  
χ 7122  
Rainbow  
MGN996  
MGN1180  
MGN1181  
MGN1182  
MGN1183

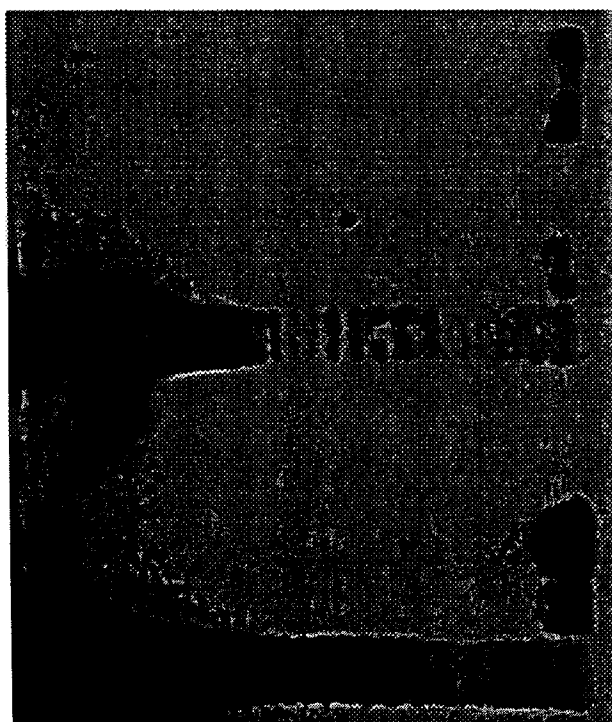


FIGURE 3A

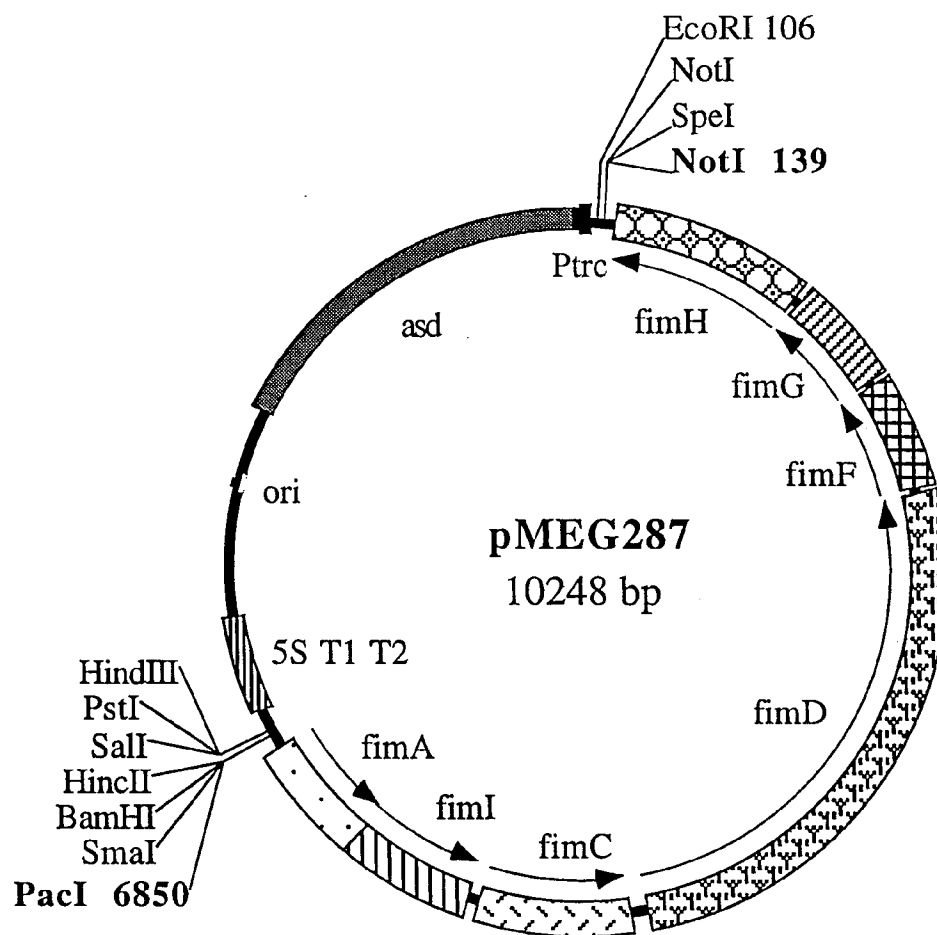
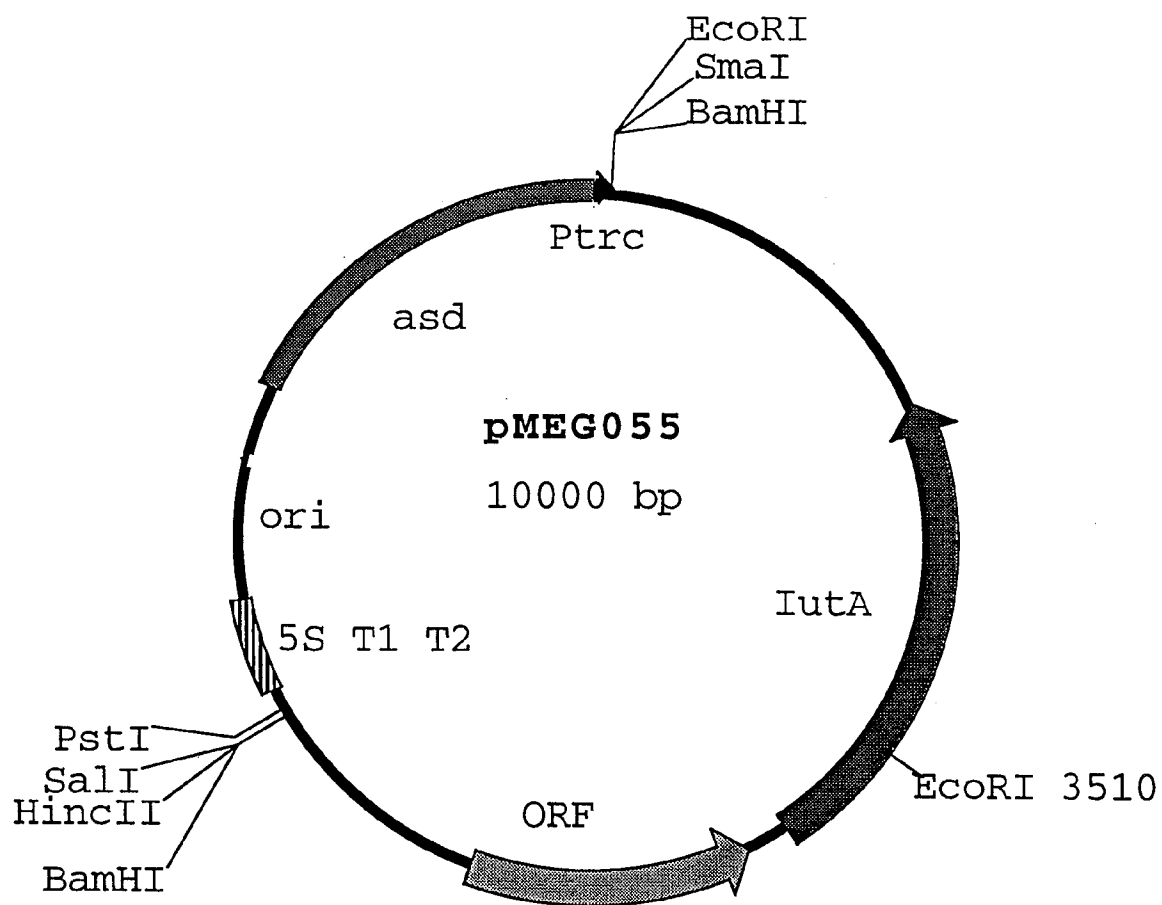


FIGURE 4

**FIGURE 5**